Supporting Information

Highly Potent and Oral Macrocyclic Peptides as a HIV-1 Protease Inhibitor: mRNA Display-Derived Hit-to-Lead Optimization

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1. Abbreviation

SPPS: solid phase peptide synthesis

Fmoc: 9-fluorenylmethyloxycarbonyl

tBu: tertiary butyl

Trt: trityl

Boc: tert-butoxycarbonyl

DIPEA: N, N-diisopropylethylamine

Et_3N: triethylamine

TFA: trifluoroacetic acid

DMF: N, N-dimethylformamide

DMSO: dimethyl sulfoxide

NMP: N-methyl-2-pyrrolidone

CH_2Cl_2: dichloromethane

$CHCl_3$: chloroform

AcOEt: ethyl acetate

MeOH: methanol

Et_2O: diethyl ether

MTBE: methyl tert-butyl ether

DOX: 1,4-dioxane

HATU: 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate

HOAt: 1-hydroxy-7-azabenzotriazole

TIPS: triisopropyl silane

DODT: 3,6-dioxa-1,8-octanedithiol

Oxyma: ethyl (hydroxyimino)cyanoacetate

DIC: N,N'-diisopropylcarbodiimide

COMU: ethyl2-cyano-2-((dimethyliminio)(morpholino)methyloxyimino)acetate hexafluorophosphate

PPh_3: triphenyl Phosphine

PPh_3O: triphenylphosphine Oxide

TMSCl: trimethylsilyl Chloride

NaI: sodium Iodide

MeLi: methyllithium

$TMSCH_2$: trimethylsilyldiazomethane

Fmoc-Cl: 9-fluorenylmethyl Chloroformate

Fmoc-OSu: N-(9-fluorenylmethoxycarbonyloxy)succinimide

$NaHCO_3$: sodium hydrogen carbonate

Na_2SO_4: sodium sulfate

HCl: hydrochloric Acid

NaOH: sodium hydroxide

K_2CO_3: potassium carbonate

$Na_2S_2O_3$: sodium Thiosulfate

^1H NMR: proton nuclear magnetic resonance

HPLC: high Performance Liquid Chromatography

UV: ultraviolet

MS: mass spectrometry

ESI: electrospray ionization

rt: room temperature

h: hour

min: minute

2. General Procedure for Synthesis of cyclic peptide

No unexpected or unusually high safety hazards were encountered. Fmoc amino acid of N-Boc protected Trp were used for peptide syntheses if necessary.

Synthetic Method S-A

SPPS was performed with Biotage Intiator+ Alstra, a fully automated peptide synthesizer. Cysteamine-bound 2-chlorotrityl resin was swelled in DMF for 30 min. The resin was drained and treated with 20% piperazine in DMF (24 mL/mmol) to deprotect Fmoc before subjected to SPPS. The resin was drained and treated with a solution of Fmoc-protected amino acid (4 equiv.), HATU (4 equiv.), HOAt (4 equiv.), dissolved in DMF (24 mL/mmol) and DIPEA (8 equiv.) dissolved in NMP (8 mL/mmol). The contents were gently agitated for 5 min at 75℃. After drained, the resin was washed with DMF. For amino acids next to *N*-methyl amino acids, double coupling was carried out. Deprotection of Fmoc was conducted by treatment with 20% piperidine in DMF (24mL/mmol) at rt for 1 x 3 min and 1 x 10 min followed by coupling of the next amino acid. The coupling and deprotection protocol were repeated to afford the elongated linear peptide. After elongation with amino acids, to the resulting peptide-bound resin was added a solution of chloroacetic acid (4 equiv.), HATU (4 equiv.) in DMF (16 mL/mmol) and DIPEA (4 equiv.) dissolved in NMP (4 mL/mmol). The mixture was agitated for 40 min at rt. After drained, the protected linear peptide was obtained.

Synthetic Method S-B

SPPS was performed with Syro I, a fully automated parallel peptide synthesizer. Cysteamine-bound 2-chlorotrityl resin was swelled in DMF for 30 min. The resin was drained and treated with 20% piperazine in DMF (24 mL/mmol) to deprotect Fmoc before subjected to SPPS. The resin was drained and treated with a pre-mixed solution of Fmoc-protected amino acid (4 equiv.), HATU (4 equiv.) dissolved in DMF (16 mL/mmol) and DIPEA (8 equiv.) dissolved in NMP (8 mL/mmol). The contents were gently agitated for 20 min at 75℃. After drained, the resin was washed with DMF for three times. For amino acids next to *N*-methyl amino acids, double coupling was carried out. Deprotection of Fmoc was conducted by treatment with 20% piperidine in DMF (24 mL/mmol) at rt for 1 x 3 min and 1 x 12 min followed by coupling of the next amino acid. The coupling and deprotection protocol were repeated to afford the elongated linear peptide. After elongation with amino acids, to the resulting peptide-bound resin was added pre-mixed chloroacetic acid (4 equiv.), HATU (4 equiv.) dissolved in DMF (16 mL/mmol) and DIPEA (4 equiv.) dissolved in NMP (4 mL/mmol). The mixture was agitated for 40 min at rt. After drained, the protected linear peptide was obtained.

Synthetic Method S-C

SPPS was performed with Liberty Blue, a fully automated parallel peptide synthesizer. Cysteamine-

bound 4-methoxytrityl resin or resin **19** was treated with 20% piperidine in DMF (24 mL/mmol) to deprotect Fmoc before subjected to SPPS. The resin was drained and treated with a solution of Fmoc-protected amino acid (5 equiv.), DIC (10 equiv.), Oxyma (5 equiv.) dissolved in DMF (40 mL/mmol). The contents were gently agitated for 2 min at 90℃. For amino acids next to *N*-methyl amino acids and Val at 4th position and α-methyl amino acid, double coupling was carried out. Deprotection of Fmoc was conducted by treatment with 20% piperidine in DMF (24 mL/mmol) for 1 min at 90℃. After drained, the resin was washed with DMF followed by coupling of the next amino acid. The coupling and deprotection protocol were repeated to afford the elongated linear peptide. After elongation with amino acids, to the resulting peptide-bound resin was added a solution of chloroacetic acid (5 equiv.), DIC (10 equiv.), Oxyma (5 equiv.) dissolved in DMF (40 mL/mmol). The mixture was agitated at rt for 30 min. After drained, the protected linear peptide was obtained.

Synthetic Method S-D

SPPS was performed with Liberty Blue, a fully automated parallel peptide synthesizer. Alanine-bound 2-chlorotrityl resin or Resin **17** was treated with 20% piperidine in DMF (24 mL/mmol) to deprotect Fmoc before subjected to SPPS. The resin was drained and treated with a solution of Fmoc-protected amino acid (5 equiv.), DIC (10 equiv.), Oxyma (5 equiv.) dissolved in DMF (40 mL/mmol). The contents were gently agitated for 10 min at 50℃. For amino acids next to *N*-methyl amino acids, double coupling was carried out. For amino acids next to α-methyl amino acid, quattro coupling was carried out. Deprotection of Fmoc was conducted by treatment with 20% piperidine in DMF (24 mL/mmol) for 1 min at 90℃. After drained, the resin was washed with DMF followed by coupling of the next amino acid. The coupling and deprotection protocol were repeated to afford the elongated linear peptide.

Cleavage from resin and cyclization: Method C-A

The protected linear peptide was added TFA/TIPS/H_2O (95/2.5/2.5, 80 mL/mmol) and the resulting mixture was shaken for 1.5 h at rt. The released linear peptide was precipitated with cold MTBE/n-hexane (1:1, 800 mL/mmol), washed with MTBE/n-hexane (1:1, 800 mL/mmol), and dried in vacuo to give the linear peptide. To a solution of the linear peptide in DMSO (200 mL/mmol) was added Et_3N (10 equiv.). After stirring for 1 h, the reaction mixture was evaporated by Genevac HT-4 evaporator to afford the cyclic peptide.

Cleavage from resin and cyclization: Method C-B

The protected linear peptide was added CH_2Cl_2/TIPS/TFA (95/4/1, 120 mL/mmol) and shaken for 1 h at rt. After filtering the mixture, the residual resin was added CH_2Cl_2/TIPS/TFA (95/4/1, 120 mL/mmol) and shaken for 30 min at rt. After filtering the residual resin, the combined filtrate was

neutralized with Et_3N (6 mL/mmol) and evaporated to give the linear peptide. The residue was added AcOEt and washed with H_2O, and evaporated. To a solution of the crude linear peptide in DMF (200 mL/mmol) was added Et_3N (10 equiv.). After stirring for 1 h or overnight, the reaction mixture was evaporated by Genevac HT-4 evaporator to afford the cyclic peptide.

Purification Method P-A

Peptides were purified using a Waters SQD system connected to a Phenomenex Kinetex C18 (5 μm, 100 Å, 30 mm x 150 mm) or Phenomenex Gemini-NX C18 AXIA (5 μm, 110 Å, 30 mm x 100 mm), reversed phase HPLC column eluting with a solvent gradient A:B, where A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile at a flow rate of 15 mL/min.

Purification Method :P-B

Peptides were purified using a Waters SQ detector 2 system connected to a Phenomenex Kinetex EVO C18 column (5 μm, 100 Å, 30 mm x 150 mm), reversed phase HPLC column eluting with a solvent gradient A:B, where A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile at a flow rate of 15 mL/min.

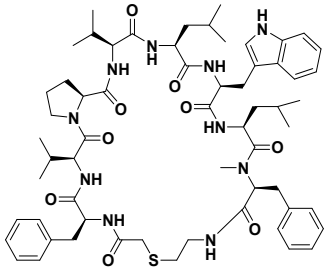
Analytical Method A-A

Peptides were analyzed using an Agilent Infinity 1290 and 6130 or 6135 LC-MS system coupled to a Kinetex EVO C18 (2.6 microns, 100 A, 2.6 mm x 50 mm) reversed phase HPLC column eluting with a solvent gradient of A:B, where A = 0.05% TFA in water and B = 0.05% TFA in acetonitrile (Gradient: as follow, Detection: UV 210 or 220nm) at a flow rate of 0.5 mL/min.

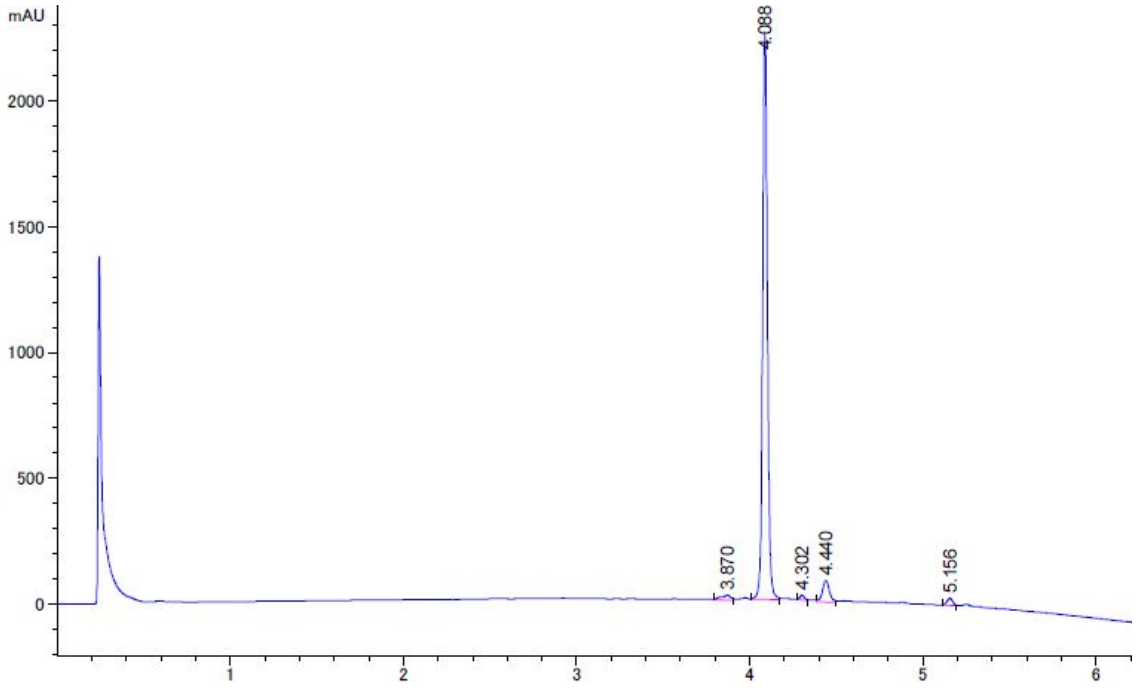
Gradient: 10-95% (6 min gradient of %B), column temp: 40 ℃

3. Synthesis of Peptides

Synthesis of peptide **1**

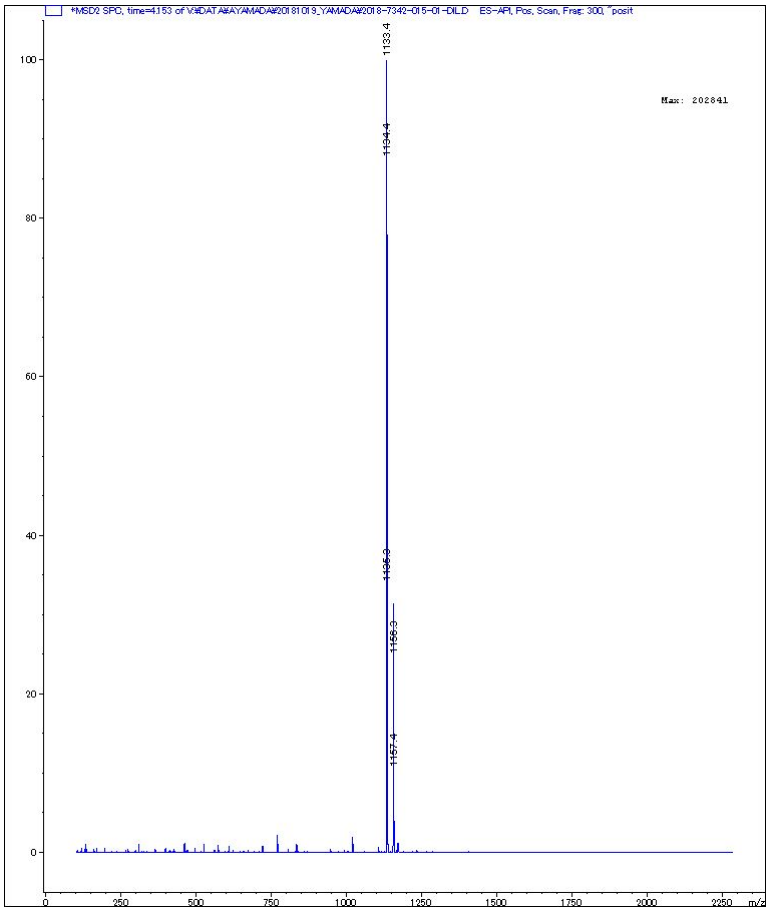


Peptide **1** was synthesized by Synthetic **Method S-A** with cysteamine-bound 2-chlorotrityl resin (1.52 mol/g, 0.1 mmol, 66 mg), and cleavage from resin and cyclization: **Method C-A**. The solution was dissolved in AcOEt (40 mL) and added H_2O (40 mL), separated with the organic layer. The aqueous layer was extracted with AcOEt (40 mL) and the combined organic layer was washed H2O, brine, dried over Na_2SO_4. After the solvent was removed under reduced pressure, the cyclic peptide was purified by flash column chromatography (First: 0-10% $CHCl_3$/MeOH, Second: 50-100% AcOEt/n-hexane). The obtained pure fractions were combined and evaporated, then triturated with MTBE/n-hexane to give 35.5 mg (y. 31%) of peptide **1** as a white solid. With Analytical **Method A-A**, UV purity (210 nm) = 90.8% (Retention time = 4.09 min): MS-ESI (m/z) calcd for C61H85N10O9S $[M+H]^+$ 1133.6, found 1133.4.

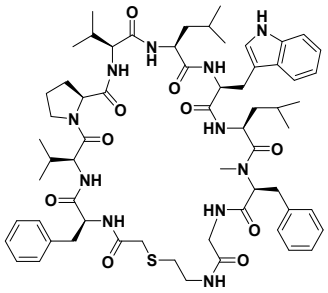


Signal 1: DAD1 A, Sig=210,4 Ref=off

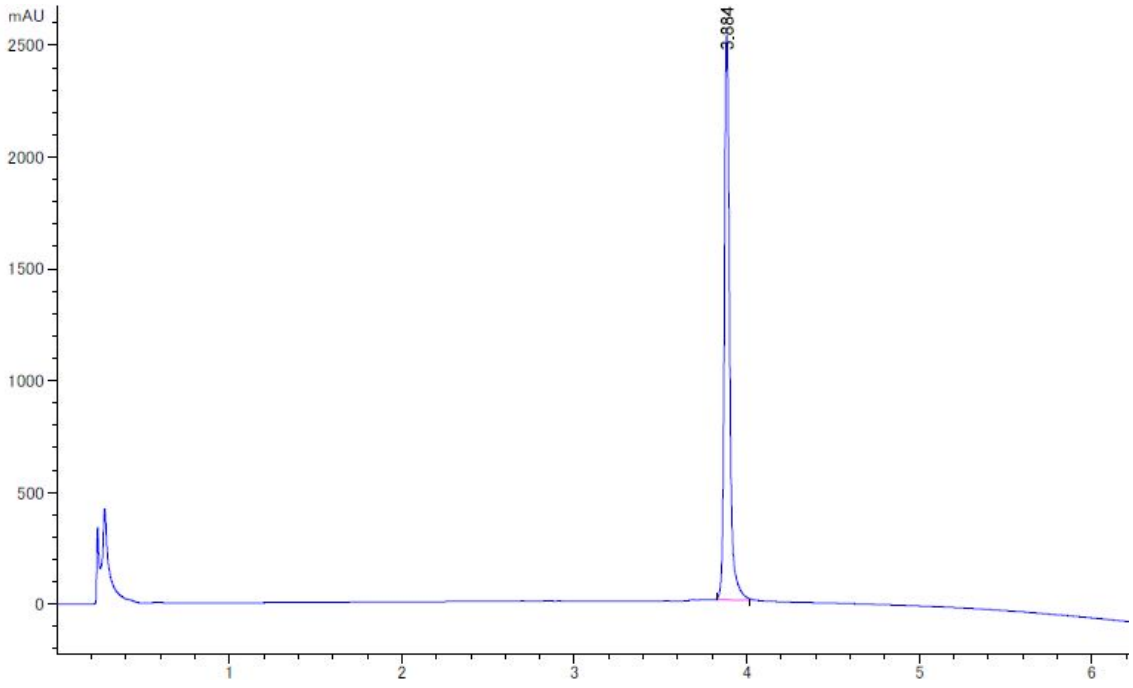
Peak #	RT [min]	Type	Width [min]	Area %	Area
1	3.870	MM	0.069	1.768	83.127
2	4.088	MM	0.032	90.833	4.270e3
3	4.302	MM	0.031	0.806	37.908
4	4.440	MM	0.046	5.139	241.579
5	5.157	MM	0.039	1.454	68.342



Synthesis of peptide **2**



Peptide **2** was synthesized by Synthetic **Method S-B** with cysteamine-bound 2-chlorotrityl resin (0.05 mmol, 33 mg), and cleavage from resin and cyclization: **Method C-A**. Peptide **2** was purified using Purification **Method P-A**. The obtained pure fractions were combined and evaporated, then lyophilized to give 15.6 mg (y. 26%) of peptide **1** as a white solid. With Analytical **Method A-A**, UV purity (210 nm) = 100% (Retention time = 3.88 min): MS-ESI (m/z) calcd for C63H88N11O10S $[M+H]^+$ 1190.6, found 1190.4.



Signal 1: DAD1 A, Sig=210,4 Ref=off

Peak #	RT [min]	Type	Width [min]	Area %	Area
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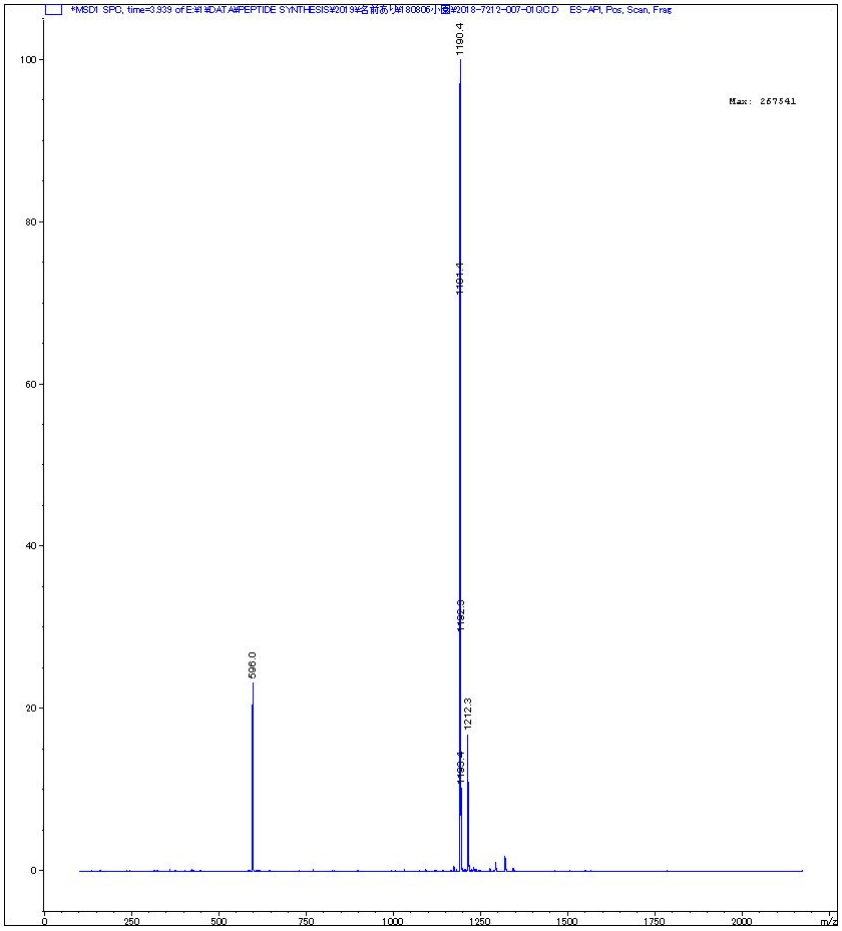
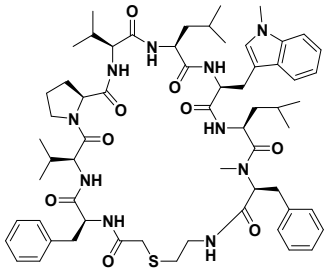
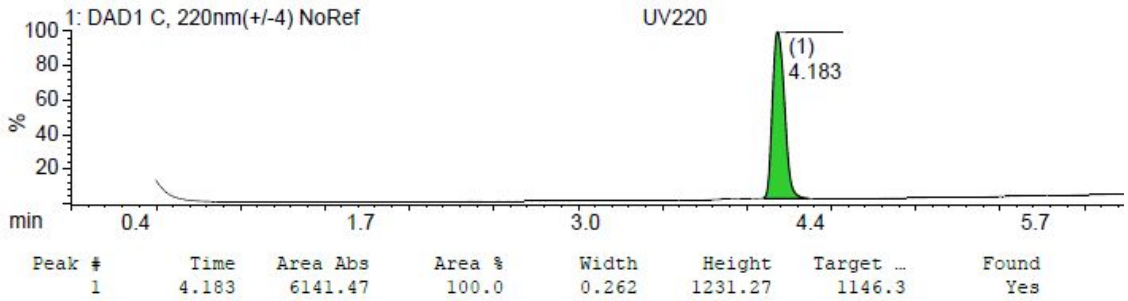

Max: 267541
1190.4
1191.4
1192.3
596.0
1212.3
1189.4
100
80
60
40
20
0
0
250
500
750
1000
1250
1500
1750
2000
m/z

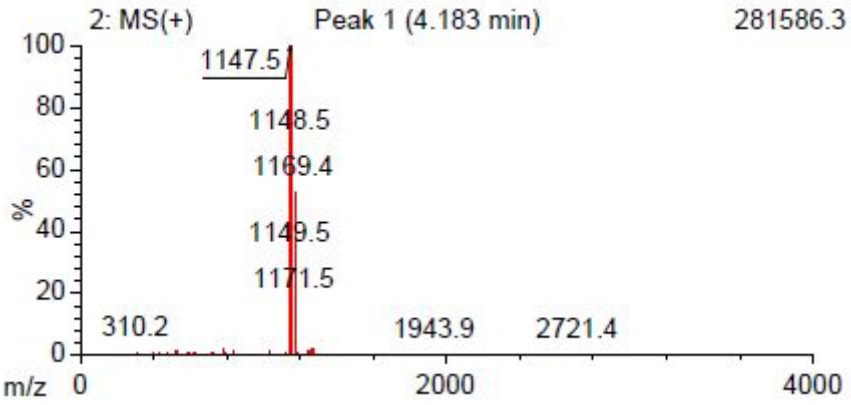
Synthesis of peptide **3**



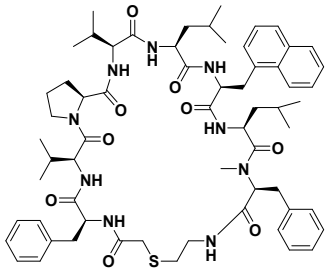
Peptide **3** was synthesized by Synthetic **Method S-B** with cysteamine-bound 2-chlorotrityl resin (0.05 mmol, 33 mg), and cleavage from resin and cyclization: **Method C-A**. Peptide **3** was purified using Purification **Method P-A**. The obtained pure fractions were combined and evaporated, then lyophilized to give 11 mg (y. 18%) of peptide **1** as a white solid. With Analytical **Method A-A**, UV purity (220 nm) = 100% (Retention time = 4.18 min): MS-ESI (m/z) calcd for C62H87N10O9S $[M+H]^+$ 1147.6, found 1147.5.



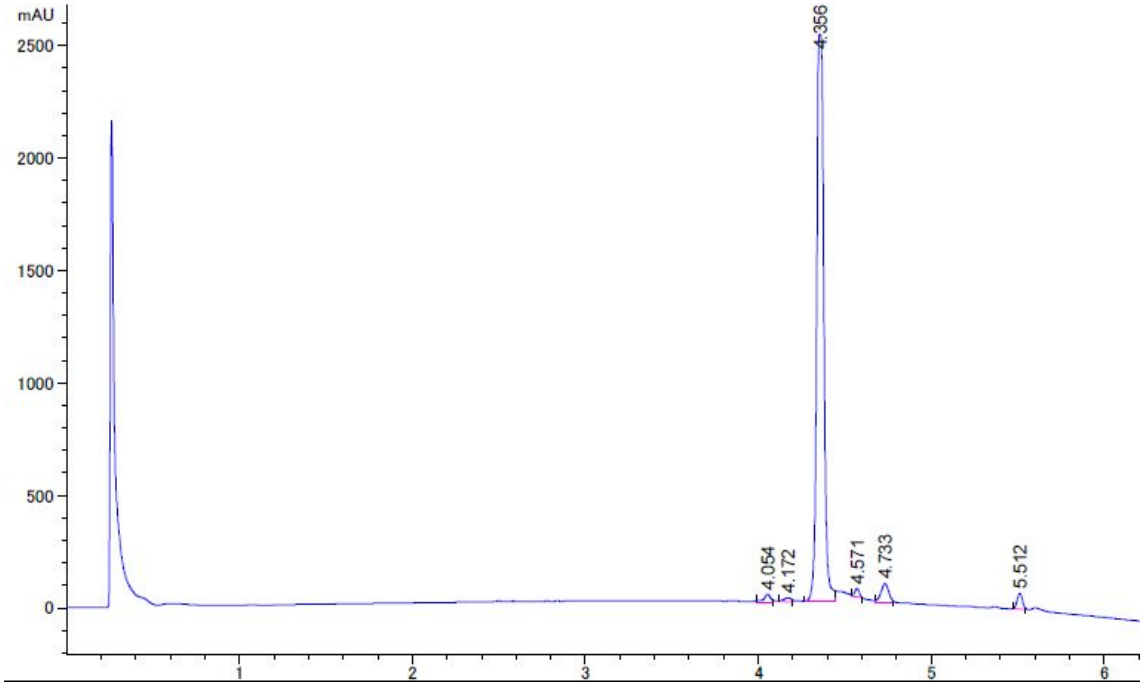
Peak #	Time	Area Abs	Area %	Width	Height	Target …	Found
1	4.183	6141.47	100.0	0.262	1231.27	1146.3	Yes



Synthesis of peptide **4**

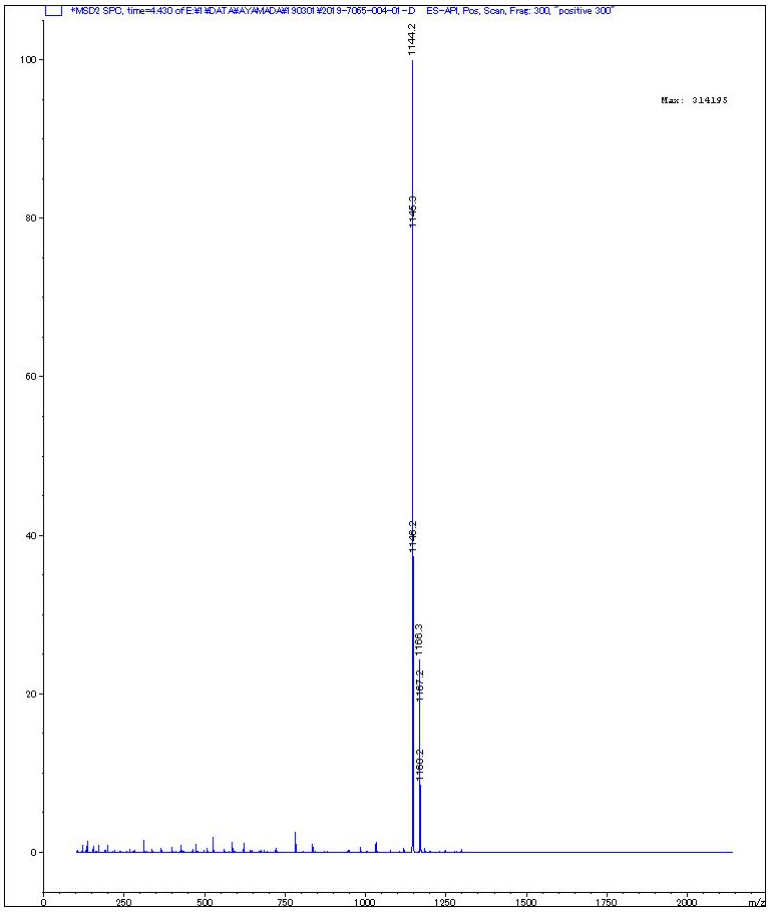


Peptide **4** was synthesized by Synthetic **Method S-A** with cysteamine-bound 2-chlorotrityl resin (0.05 mmol, 33 mg) and, cleavage from resin and cyclization: **Method C-A**. For amino acids next to proline, single coupling was carried out for 10 min at 75℃. For amino acids next to *N*-methyl phenyl alanine, double coupling was carried out for 10 min at 75 ℃. Peptide **4** was purified by flash column chromatography (0-5% $CHCl_3$/MeOH). The obtained pure fractions were combined and evaporated to give 27.7 mg (y. 49%) of peptide **1** as a white solid. With Analytical **Method A-A**, UV purity (210 nm) = 92.1% (Retention time = 4.36 min): MS-ESI (m/z) calcd for C63H86N9O9S $[M+H]^+$ 1144.6, found 1144.2.

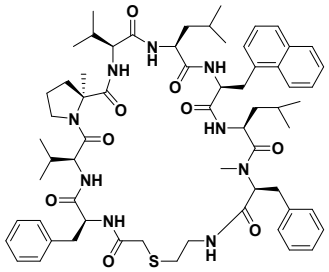


Signal 1: DAD1 A, Sig=210,4 Ref=off

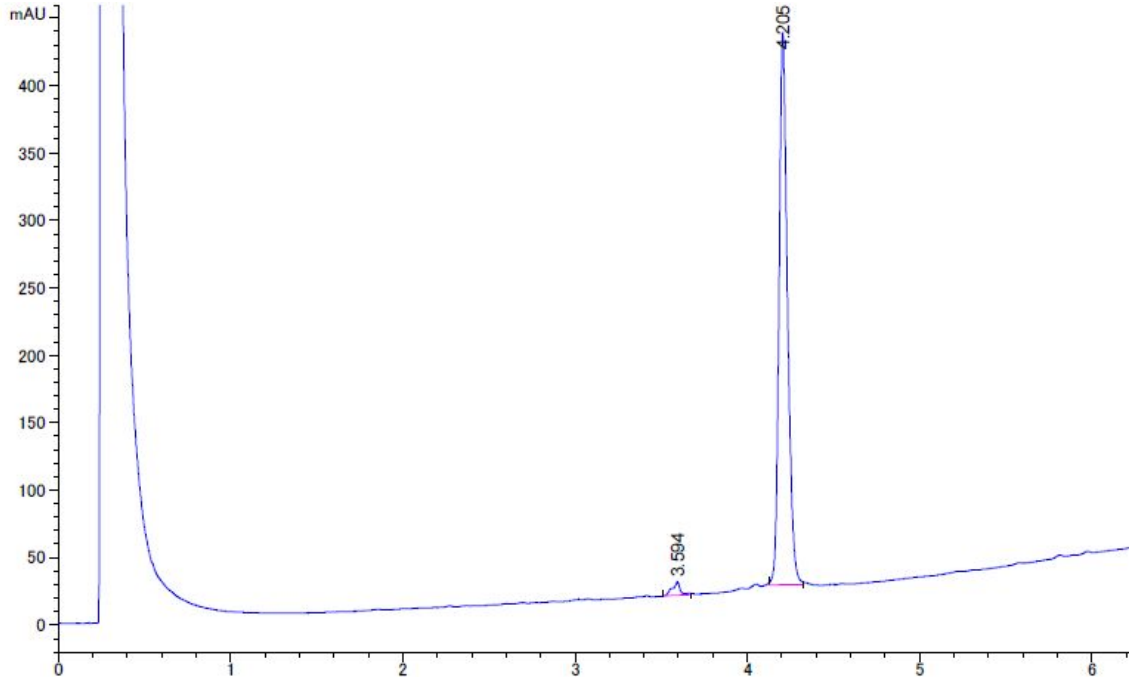
Peak #	RT [min]	Type	Width [min]	Area %	Area
1	4.054	MM	0.045	1.177	91.577
2	4.173	MM	0.049	0.674	52.472
3	4.356	MM	0.047	92.102	7.165e3
4	4.571	MM	0.030	0.898	69.890
5	4.733	MM	0.051	3.222	250.650
6	5.512	MM	0.035	1.926	149.854



Synthesis of peptide **5**



Peptide **5** was synthesized by Synthetic **Method S-C** with cysteamine-bound 4-methoxytrityl resin (0.05 mmol, 54 mg), and cleavage from resin and cyclization: **Method C-B**. Peptide **5** was purified using Purification **Method P-A**. The obtained pure fractions were combined and evaporated, then lyophilized to give 5.6 mg (y. 10%) of peptide **5** as a white solid. With Analytical **Method A-A**, UV purity (220 nm) = 97.7% (Retention time = 4.21 min): MS-ESI (m/z) calcd for C64H88N9O9S $[M+H]^+$ 1158.6, found 1159.3.



Signal 1: DAD1 A, Sig=220,4 Ref=360,100

Peak #	RT [min]	Type	Width [min]	Area %	Area
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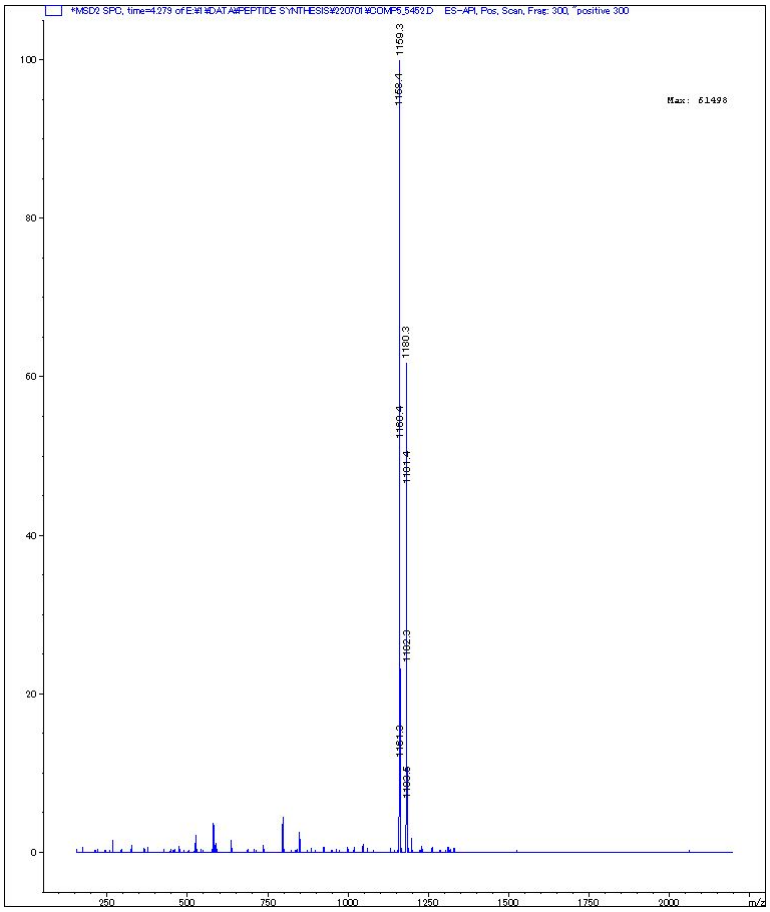
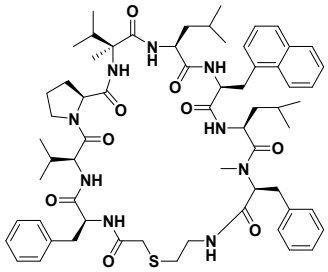
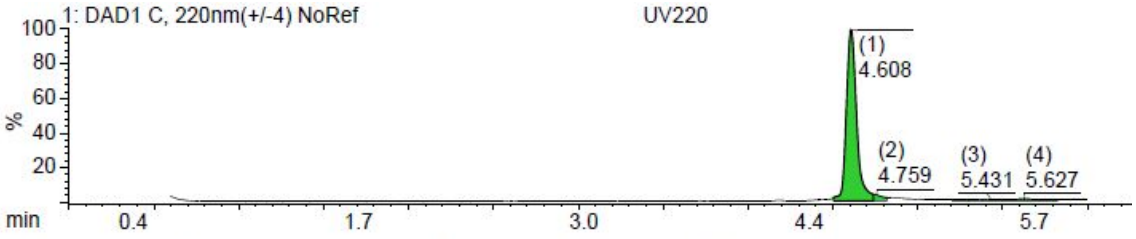
2	4.205	BB	0.051	97.742	1.395e3


+MSD2 SPC, time=4.279 of E:\#DATA\PEPTIDE SYNTHESIS\220701\COMP5_5452.D ES-API, Pos, Scan, Frag: 300, "positive 300
Max: 51498
1159.3
1160.3
1161.4
1162.3
m/z

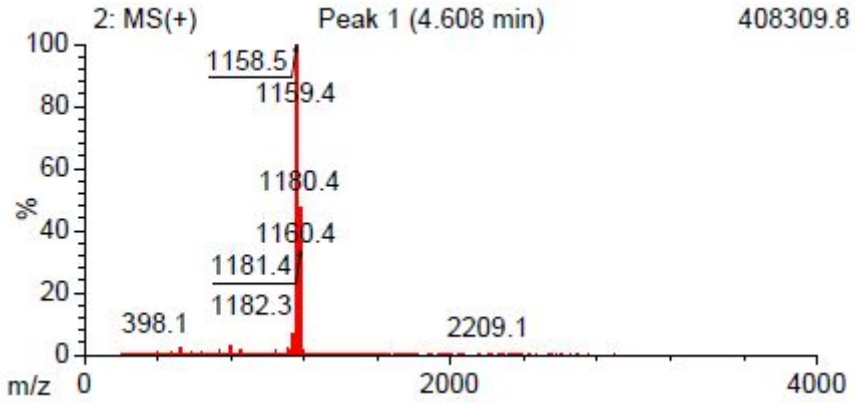
Synthesis of peptide **6**



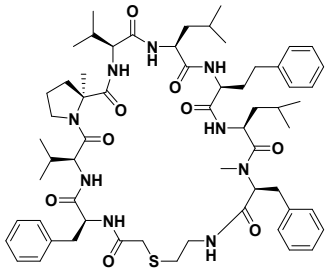
Peptide **6** was synthesized with Liberty Prime, a fully automated parallel peptide synthesizer. Cysteamine-bound 4-methoxytrityl resin (0.05 mmol, 54 mg) was treated with a solution of Fmoc-protected amino acid (5 equiv.), DIC (10 equiv.), Oxyma (5 equiv.) dissolved in DMF (40 mL/mmol). The contents were gently agitated for 2 min at 90 ℃. For amino acids next to *N*-methyl amino acids and Val at 4th position and α-methyl amino acid, double coupling was carried out. Deprotection of Fmoc was conducted by treatment with 25% pyrrolidine in DMF (10 mL/mmol) for 1 min at 90 ℃. After drained, the resin was washed with DMF followed by coupling of the next amino acid. The coupling and deprotection protocol were repeated to afford the elongated linear peptide. After elongation with amino acids, to the resulting peptide-bound resin was added a solution of chloroacetic acid (5 equiv.), DIC (10 equiv.), Oxyma (5 equiv.) dissolved in DMF (40 mL/mmol). The mixture was agitated for 30 min at rt. After drained, the protected linear peptide was subjected to cleavage from resin and cyclization: **Method C-B**. Peptide **6** was purified using Purification **Method P-A**. The obtained pure fractions were combined and evaporated, then lyophilized to give 7 mg (y. 24%) of peptide **6** as a white solid. With Analytical **Method A-A**, UV purity (220 nm) = 89.4% (Retention time = 4.61 min): MS-ESI (m/z) calcd for C64H88N9O9S $[M+H]^+$ 1158.6, found 1158.5.



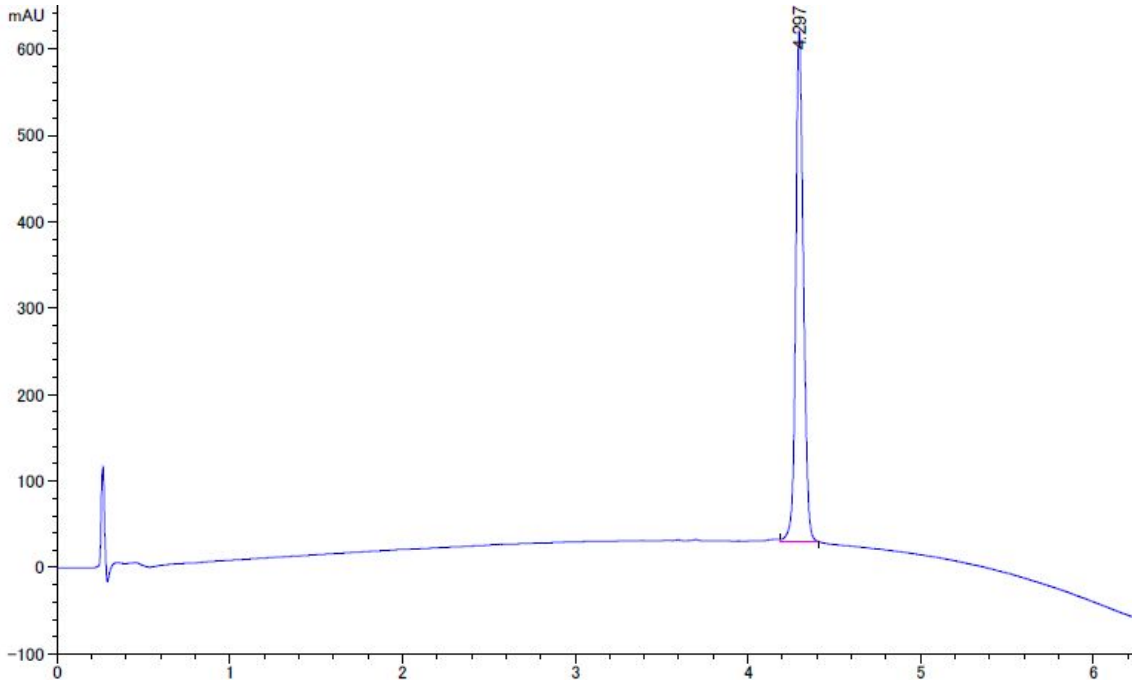
Peak #	Time	Area Abs	Area %	Width	Height	Target …	Found
1	4.608	12070.49	89.4	0.233	2882.93	1157.6	Yes
2	4.759	395.69	2.9	0.080	100.78	1157.6	Yes
3	5.431	552.86	4.1	0.361	30.92	1157.6	Yes
4	5.627	489.67	3.6	0.260	36.73	1157.6	Yes


2: MS(+)
Peak 1 (4.608 min)
408309.8
100
80
60
40
20
0
%
1158.5
1159.4
1180.4
1160.4
1181.4
1182.3
398.1
2209.1
m/z
0
2000
4000

Synthesis of peptide **7**

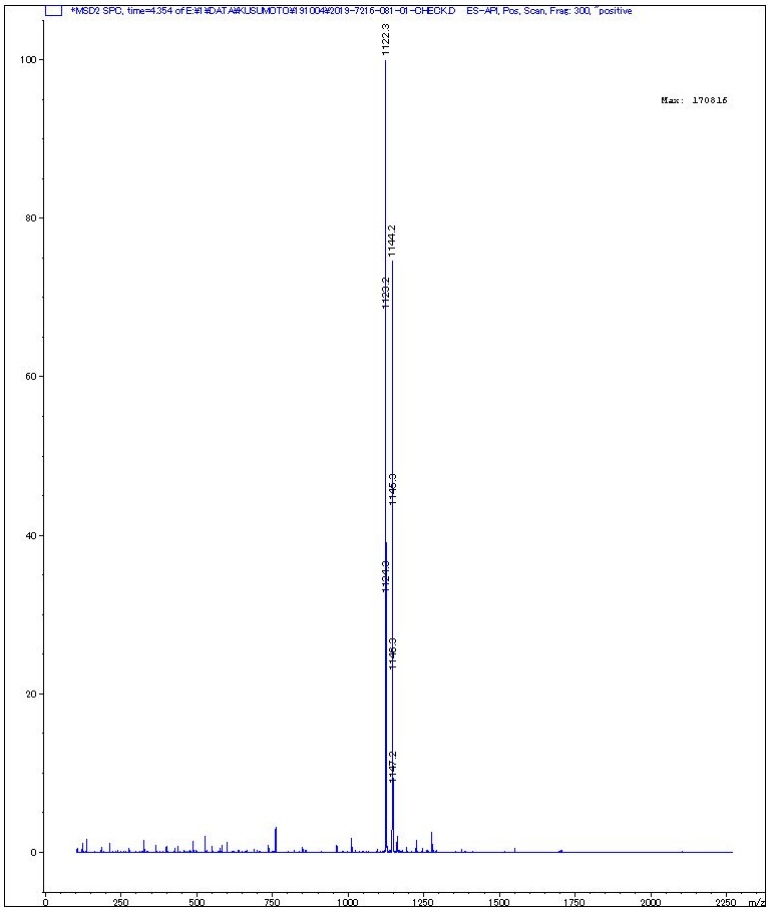


Peptide **7** was synthesized by Synthetic **Method S-C** with cysteamine-bound 4-methoxytrityl resin (0.05 mmol, 54 mg), and cleavage from resin and cyclization: **Method C-B**. Peptide **7** was purified using Purification **Method P-A**. The obtained pure fractions were combined and evaporated, then lyophilized to give 31 mg (y. 56%) of peptide **7** as a white solid. With Analytical **Method A-A**, UV purity (210 nm) = 100% (Retention time = 4.3 min): MS-ESI (m/z) calcd for $C_{61}H_{88}N_9O_9S$ $[M+H]^+$ 1122.6, found 1122.3.

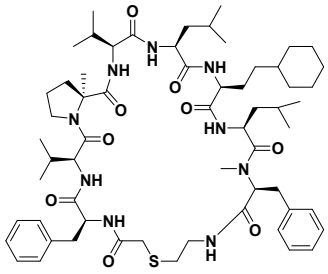


Signal 1: DAD1 A, Sig=210,4 Ref=off

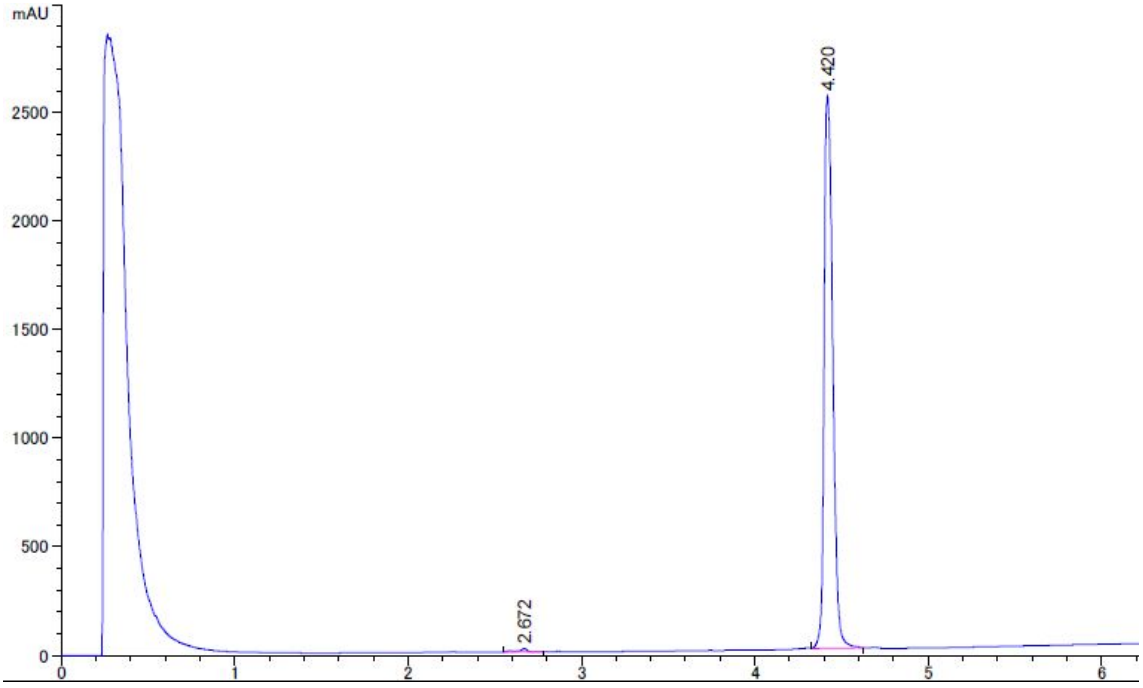
Peak #	RT [min]	Type	Width [min]	Area %	Area
1	4.297	FM	0.051	100.000	1.828e3


+MSD2 SPC, time=4.354 of E:\#DATA\KUSUMOTO\#131004\2019-7216-081-01-CHECK.D ES-API, Pos, Scan, Frag: 300, " positive
Max: 170816
1122.3
1144.2
1123.2
1146.3
1124.2
1148.3
1147.2
100
80
60
40
20
0
0
250
500
750
1000
1250
1500
1750
2000
2250
m/z

Synthesis of peptide **8**

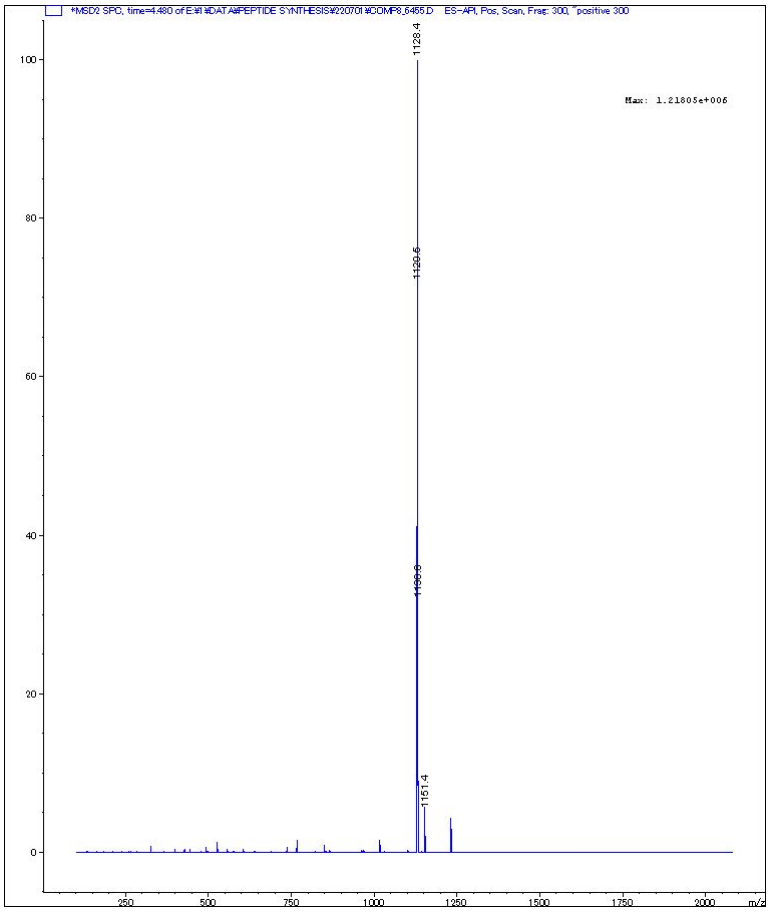


Peptide **8** was synthesized by Synthetic **Method S-C** with cysteamine-bound 4-methoxytrityl resin (0.05 mmol, 54 mg), and cleavage from resin and cyclization: **Method C-B**. Peptide **8** was purified using Purification **Method P-A**. The obtained pure fractions were combined and evaporated, then lyophilized to give 21.6 mg (y. 38%) of peptide **8** as a white solid. With Analytical **Method A-A**, UV purity (220 nm) = 99.3% (Retention time = 4.42 min): MS-ESI (m/z) calcd for C61H94N9O9S $[M+H]^+$ 1128.6, found 1128.4.

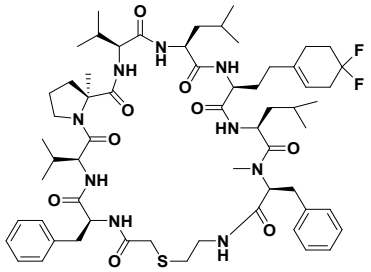


Signal 1: DAD1 A, Sig=220,4 Ref=360,100

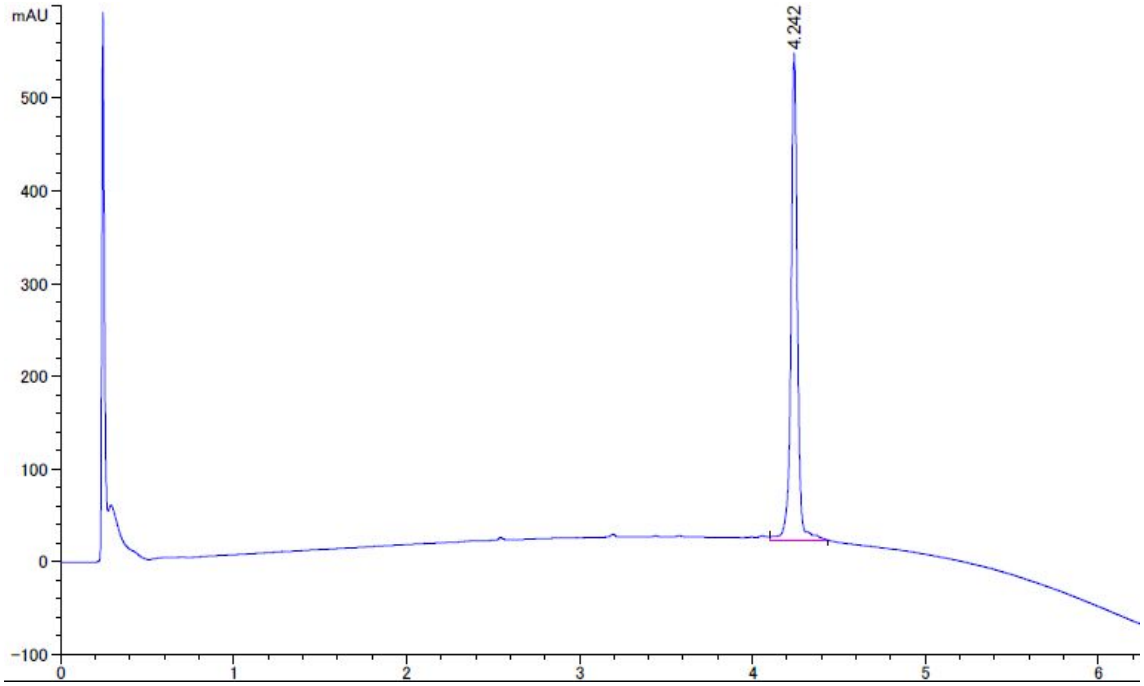
Peak #	RT [min]	Type	Width [min]	Area %	Area
1	2.672	BB	0.056	0.740	65.636
2	4.420	BB	0.055	99.260	8.801e3


+MSD2 SPC, time=4.480 of E:\#DATA\PEPTIDE SYNTHESIS\220701\COMP8_6455.D ES-API, Pos, Scan, Frag: 300, "positive 300
Max: 1.21805e+006
1128.4
1129.6
1130.6
1151.4
100
80
60
40
20
0
250
500
750
1000
1250
1500
1750
2000
m/z

Synthesis of peptide **9**

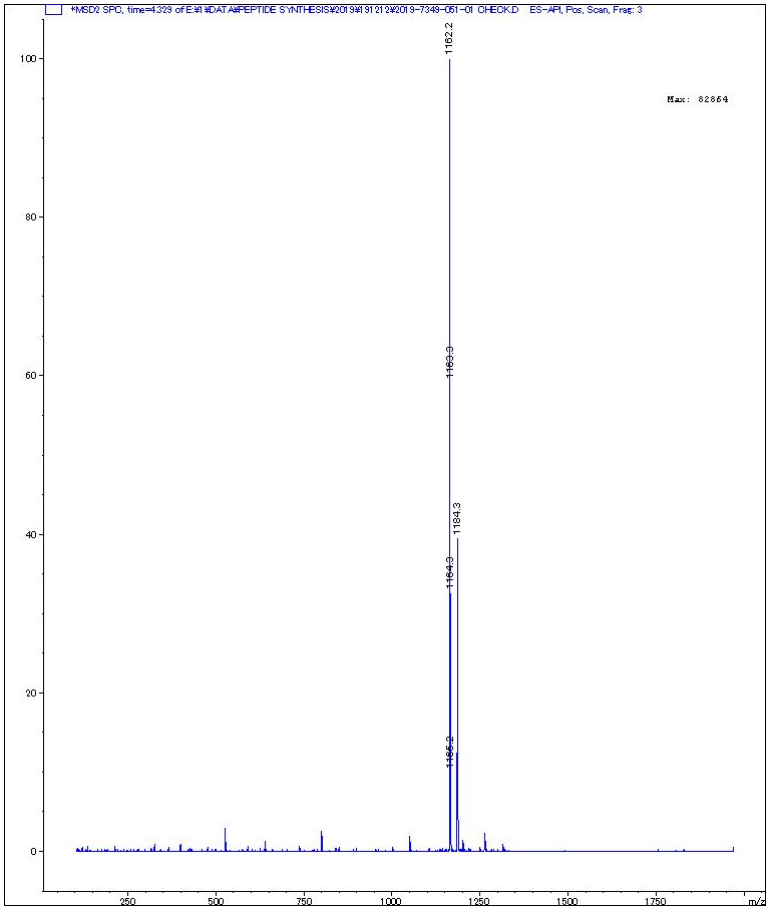


Peptide **9** was synthesized by Synthetic **Method S-C** with cysteamine-bound 4-methoxytrityl resin (0.05 mmol, 54 mg), and cleavage from resin and cyclization: **Method C-B**. For amino acids next to Val at 4th position and α-methyl amino acid, double coupling was carried out for 4 min at 90℃. Peptide **9** was purified using Purification **Method P-A**. The obtained pure fractions were combined and evaporated, then lyophilized to give 29 mg (y. 50%) of peptide **9** as a white solid. With Analytical **Method A-A**, UV purity (210 nm) = 100% (Gradient: retention time = 4.24 min): MS-ESI (m/z) calcd for C61H90F2N9O9S $[M+H]^+$ 1162.6, found 1162.2.

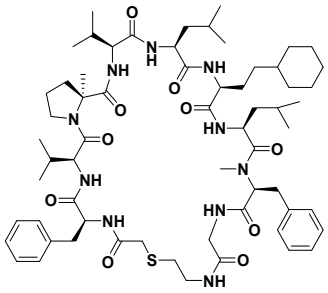


Signal 1: DAD1 A, Sig=210,4 Ref=off

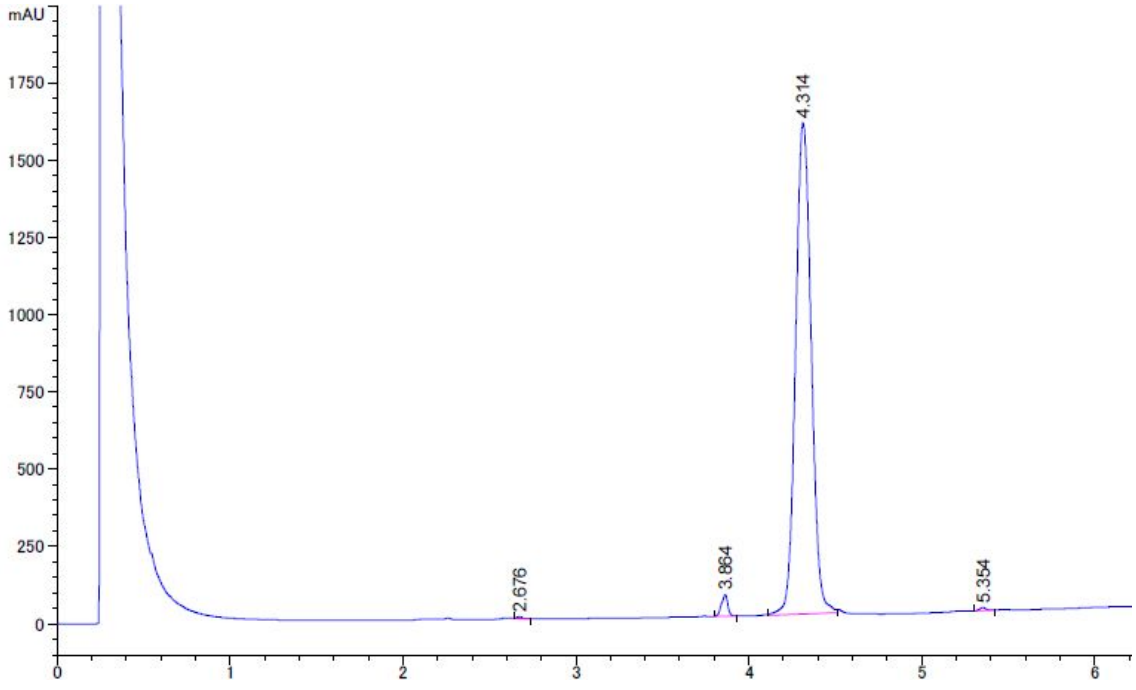
Peak #	RT [min]	Type	Width [min]	Area %	Area
1	4.242	FM	0.043	100.000	1.349e3



Synthesis of peptide **10**

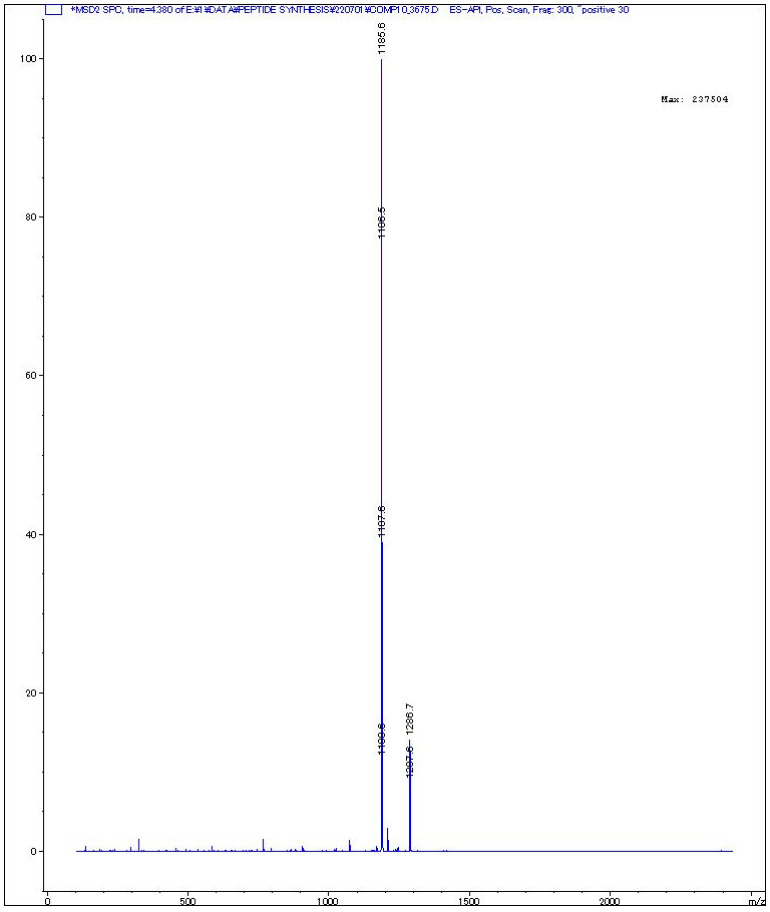


Peptide **10** was synthesized by Synthetic **Method S-C** with cysteamine-bound 4-methoxytrityl resin (0.05 mmol, 54 mg), and cleavage from resin and cyclization: **Method C-B**. For amino acids next to Val at 4th position and α-methyl amino acid, double coupling was carried out for 4 min at 90℃. Peptide **10** was purified using Purification **Method P-A**. The obtained pure fractions were combined and evaporated, then lyophilized to give 19.4 mg (y. 32%) of peptide **10** as a white solid. With Analytical **Method A-A**, UV purity (220 nm) = 97.8% (Gradient: retention time = 4.31 min): MS-ESI (m/z) calcd for C63H97N10O10S $[M+H]^+$ 1185.7, found 1185.6.

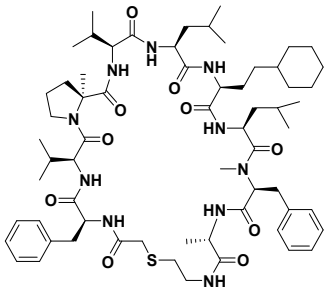


Signal 1: DAD1 A, Sig=220,4 Ref=360,100

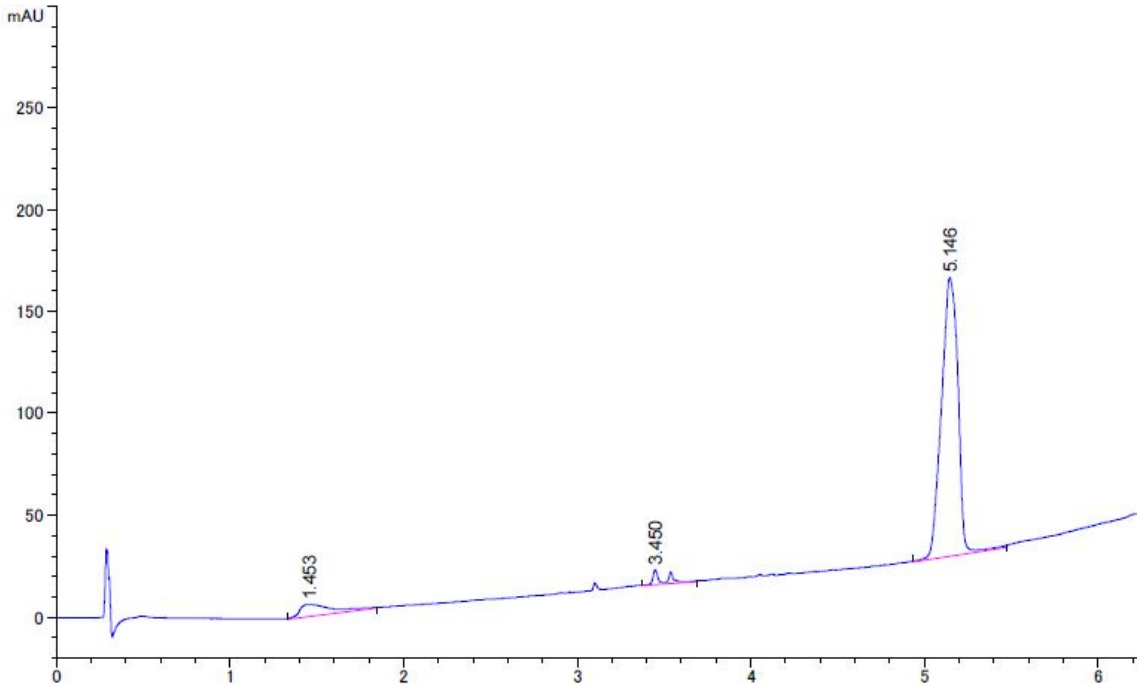
Peak #	RT [min]	Type	Width [min]	Area %	Area
1	2.676	BB	0.036	0.209	21.316
2	3.864	BB	0.036	1.730	176.283
3	4.314	BB	0.098	97.833	9.967e3
4	5.354	BB	0.038	0.227	23.164



Synthesis of peptide **11**

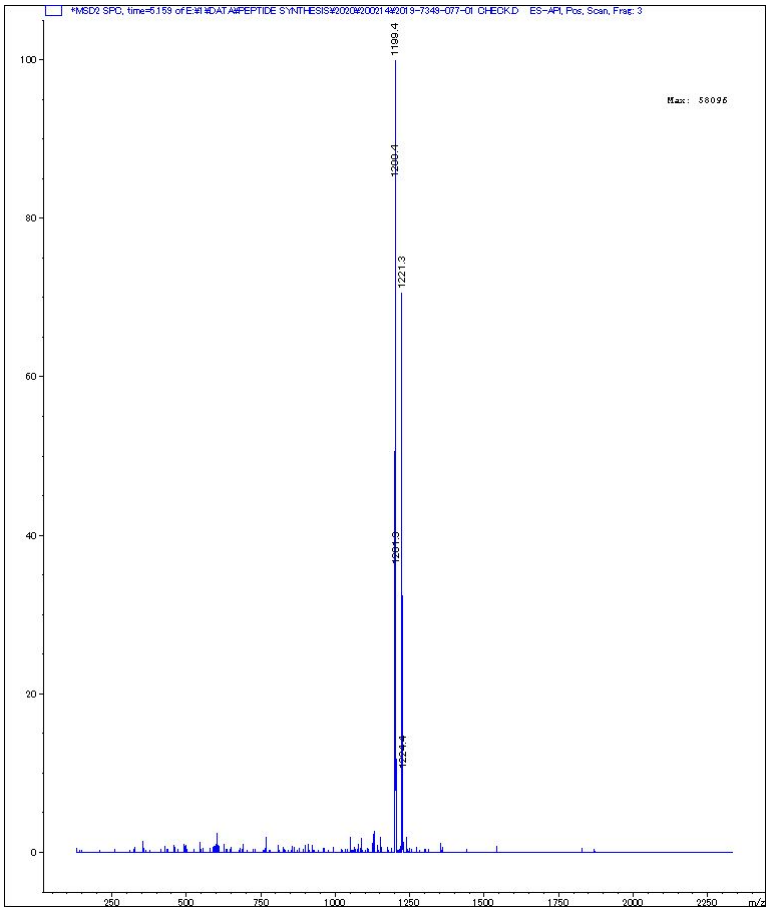


Peptide **11** was synthesized by Synthetic **Method S-C** with cysteamine-bound 4-methoxytrityl resin (0.05 mmol, 54 mg) and a peptide cleavage from resin and cyclization **Method C-B**. For amino acids next to Val at 4th position and α-methyl amino acid, double coupling was carried out for 4 min at 90 ℃. Peptide **11** was purified using Purification **Method P-A**. The obtained pure fractions were combined and evaporated, then lyophilized to give 9.7 mg (y. 16%) of peptide **11** as a white solid. With Analytical **Method A-A**, UV purity (220 nm) = 89.7% (Gradient: retention time = 5.15 min): MS-ESI (m/z) calcd for C64H99N10O10S $[M+H]^+$ 1199.7, found 1199.4.

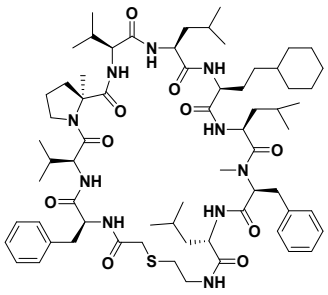


Signal 1: DAD1 A, Sig=220,4 Ref=off

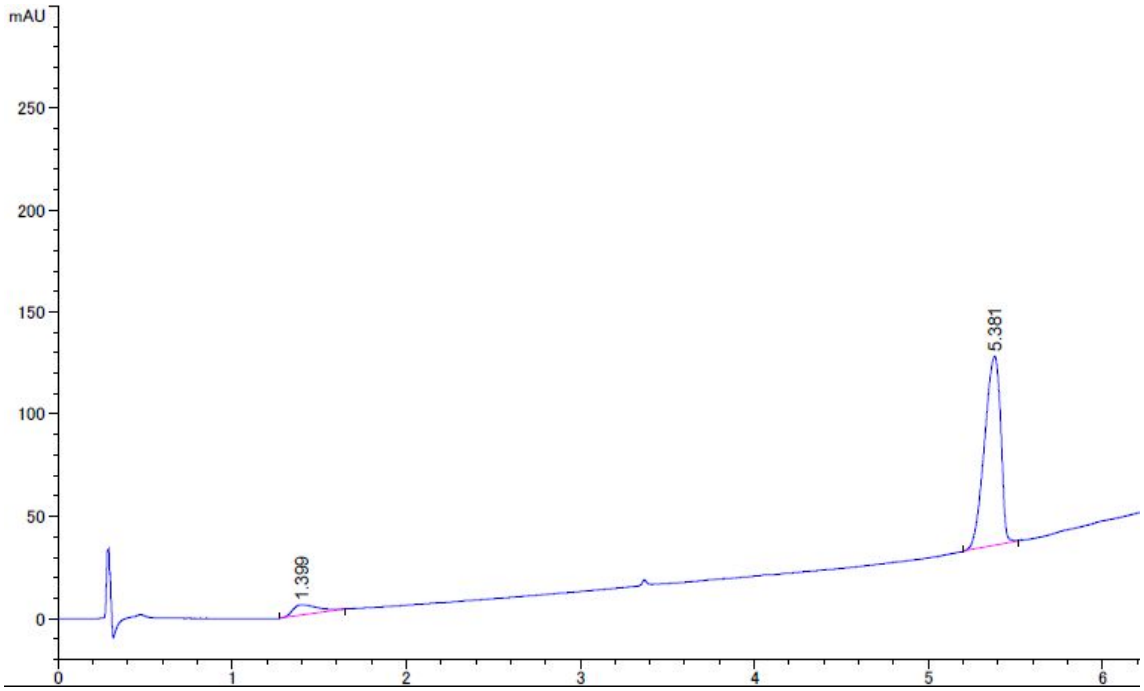
Peak #	RT [min]	Type	Width [min]	Area %	Area
1	1.453	MM	0.148	7.526	75.795
2	3.450	MM	0.062	2.735	27.546
3	5.146	MM	0.110	89.739	903.768



Synthesis of peptide **12**

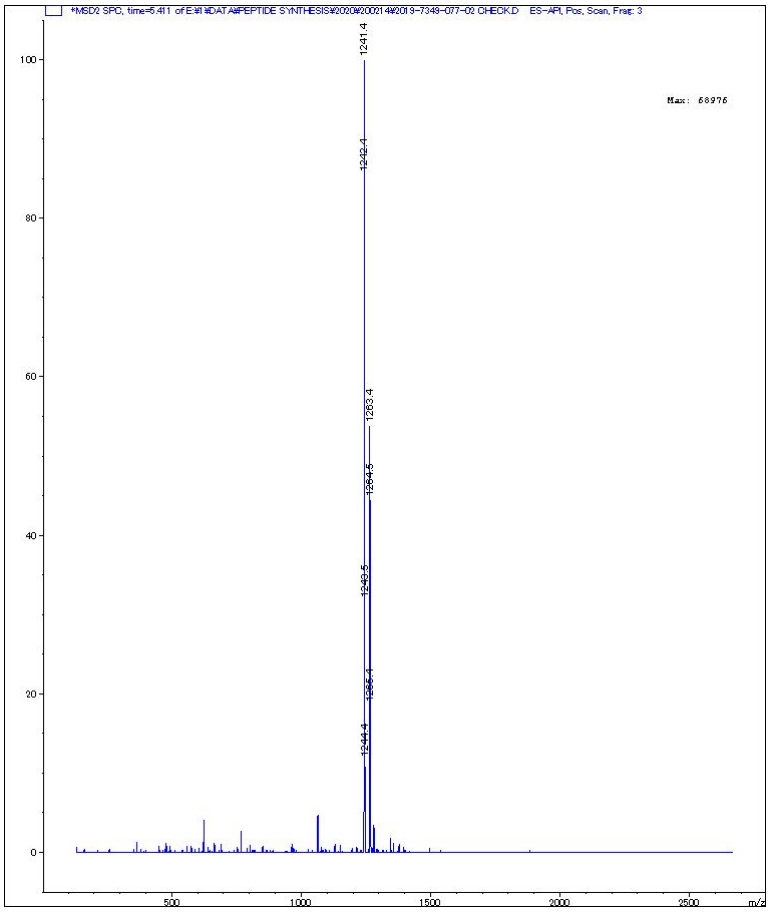


Peptide **12** was synthesized by Synthetic **Method S-C** with cysteamine-bound 4-methoxytrityl resin (0.05 mmol, 54 mg), and cleavage from resin and cyclization: **Method C-B**. For amino acids next to Val at 4th position and α-methyl amino acid, double coupling was carried out for 4 min at 90 ℃. Peptide **12** was purified using Purification **Method P-A**. The obtained pure fractions were combined and evaporated, then lyophilized to give 9.9 mg (y. 16%) of peptide **12** as a white solid. With Analytical **Method A-A**, UV purity (220 nm) = 92.3% (Gradient: retention time = 5.38 min): MS-ESI (m/z) calcd for C67H105N10O10S $[M+H]^+$ 1241.7, found 1241.4.

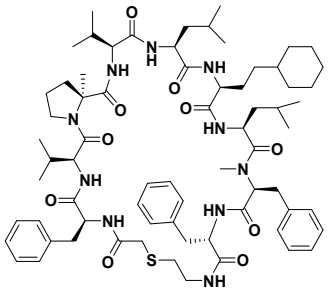


Signal 1: DAD1 A, Sig=220,4 Ref=off

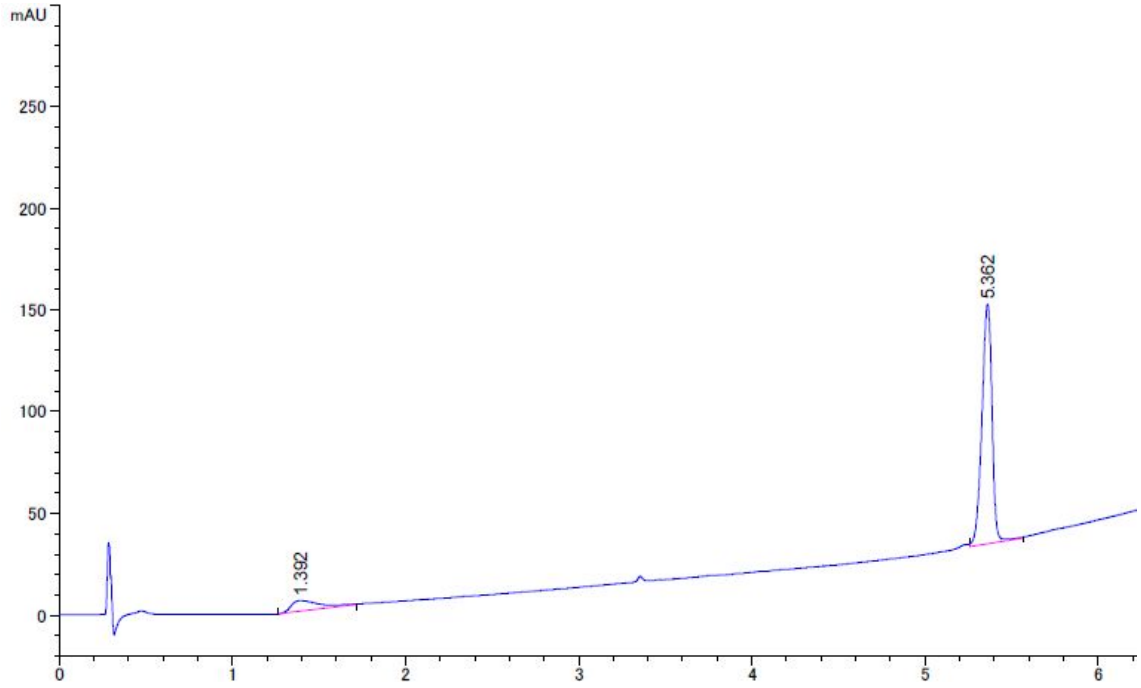
Peak #	RT [min]	Type	Width [min]	Area %	Area
1	1.399	MM	0.116	7.691	49.672
2	5.381	MM	0.107	92.309	596.140



Synthesis of peptide **13**

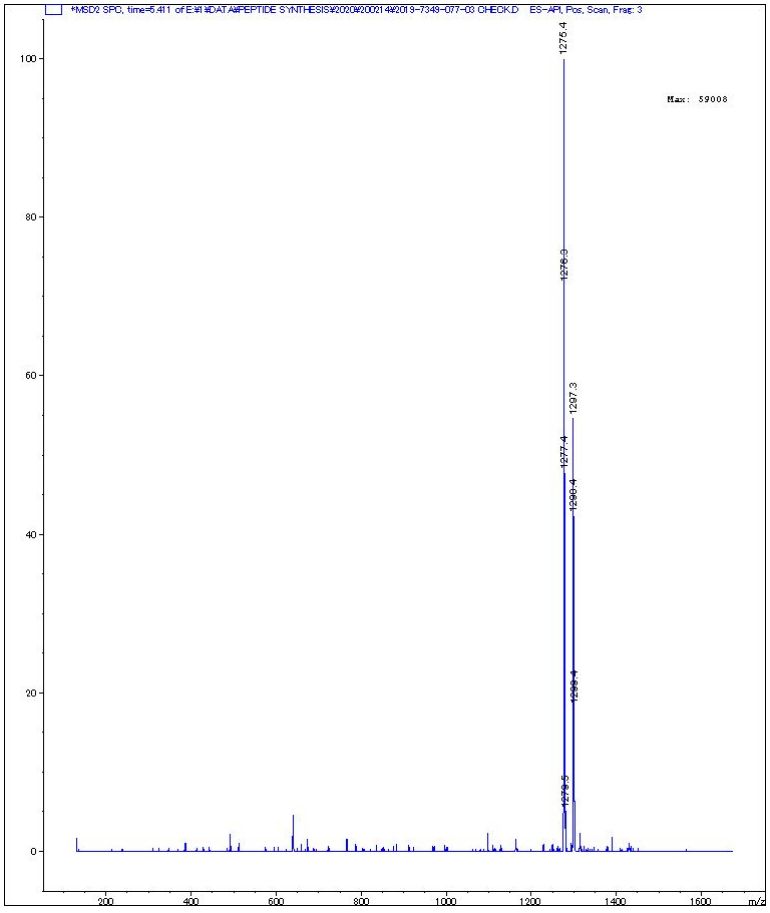


Peptide **13** was synthesized by Synthetic **Method S-C** with cysteamine-bound 4-methoxytrityl resin (0.05 mmol, 54 mg), and cleavage from resin and cyclization: **Method C-B**. For amino acids next to Val at 4th position and α-methyl amino acid, double coupling was carried out for 4 min at 90 ℃. Peptide **13** was purified using Purification **Method P-A**. The obtained pure fractions were combined and evaporated, then lyophilized to give 6.9 mg (y. 11%) of peptide **13** as a white solid. With Analytical **Method A-A**, UV purity (220 nm) = 89.3% (Gradient: retention time = 5.36 min): MS-ESI (m/z) calcd for C70H103N10O10S $[M+H]^+$ 1275.7, found 1275.4.

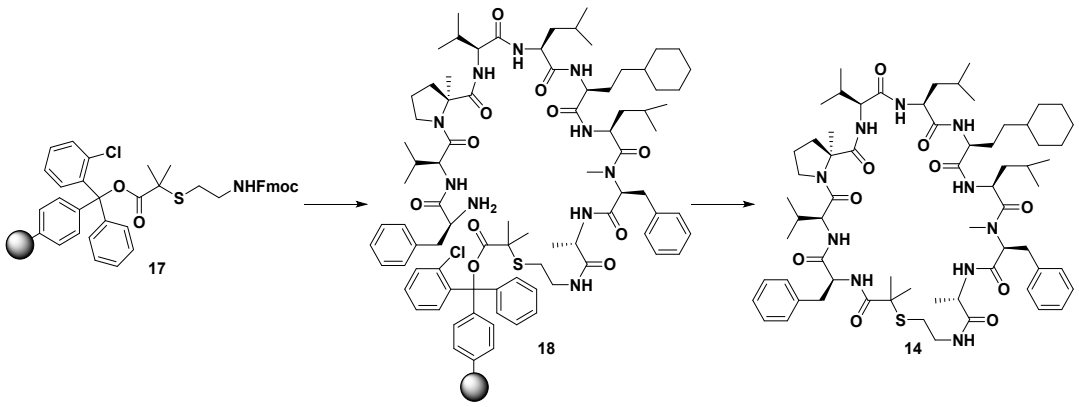


Signal 1: DAD1 A, Sig=220,4 Ref=off

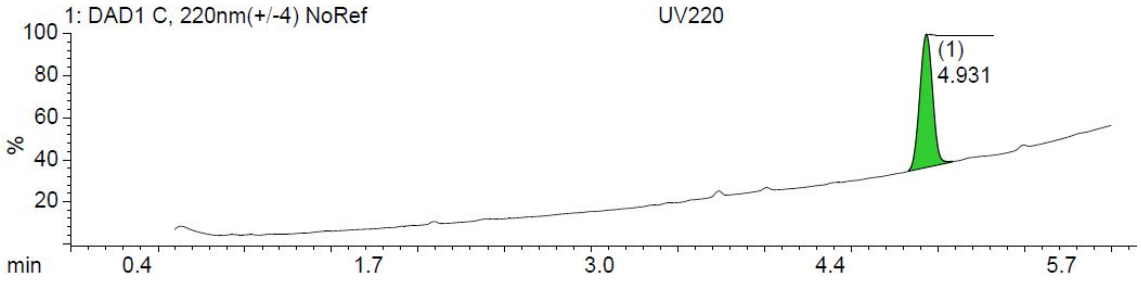
Peak #	RT [min]	Type	Width [min]	Area %	Area
1	1.392	MM	0.129	10.651	56.777
2	5.362	FM	0.067	89.349	476.310



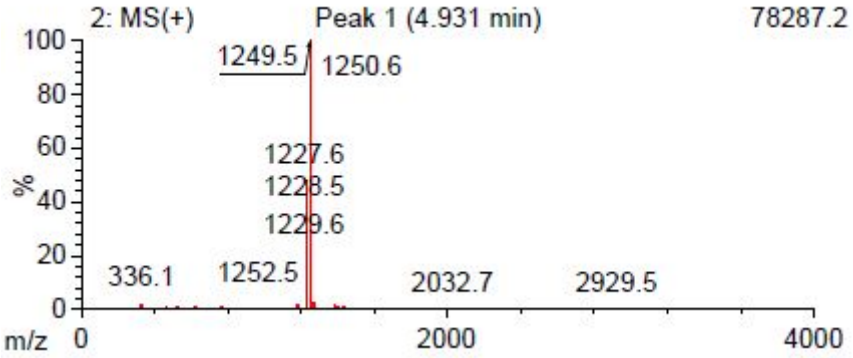
Synthesis of peptide **14**



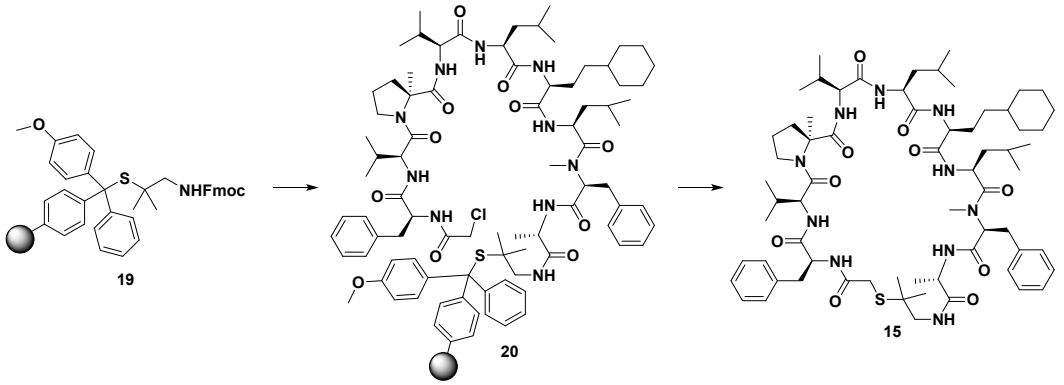
Peptide **14** was synthesized by Synthetic **Method S-D** with resin **17** (0.56 mol/g, 0.05 mmol, 54 mg). The protected linear peptide **18** was added CH_2Cl_2/TIPS/TFA (88/2/10, 20 mL/mmol) and shaken for 60 min at rt. After filtering the mixture, the residual resin was added CH_2Cl_2/TIPS/TFA (88/2/10, 20 mL/mmol) and shaken at rt for 30 min. After filtering the residual resin, the combined filtrate was neutralized with Et_3N (6 mL/mmol) and evaporated to give the linear peptide. The residue was dissolved in CH_2Cl_2 (3 ml) and added DIPEA (87 µL, 0.5 mmol), COMU (43 mg, 0.05 mmol). After being stirred for 30 min at rt, the mixture was added COMU (43 mg, 0.05 mmol). After being stirred for 30 min at rt, the mixture was added AcOEt, H_2O. The aqueous layer was extracted with AcOEt and separated with the organic layer. After the solvent was removed under reduced pressure, the cyclic peptide was purified using Purification **Method P-B**. The obtained pure fractions were combined and evaporated, then lyophilized to give 4 mg (y. 7%) of peptide 14 as a white solid. With Analytical **Method A-A**, UV purity (220 nm) = 100% (Gradient: retention time = 4.93 min): MS-ESI (m/z) calcd for C66H102N10O10SNa $[M+Na]^+$ 1249.7, found 1249.5.



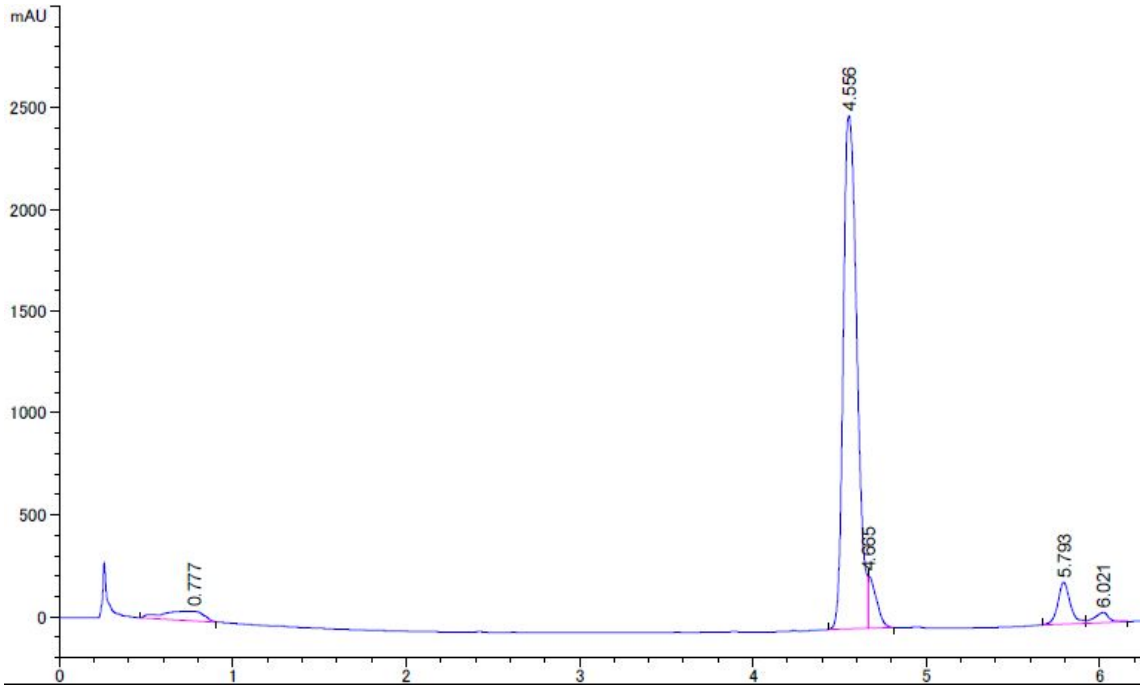
Peak #	Target …	Time	Area Abs	Area %	Width	Height	Found
1	1226.8	4.931	342.56	100.0	0.256	66.61	Yes


2: MS(+)
Peak 1 (4.931 min)
78287.2
100
80
60
%
40
20
0
1249.5
1250.6
1227.6
1228.5
1229.6
336.1
1252.5
2032.7
2929.5
m/z
0
2000
4000

Synthesis of peptide **15**

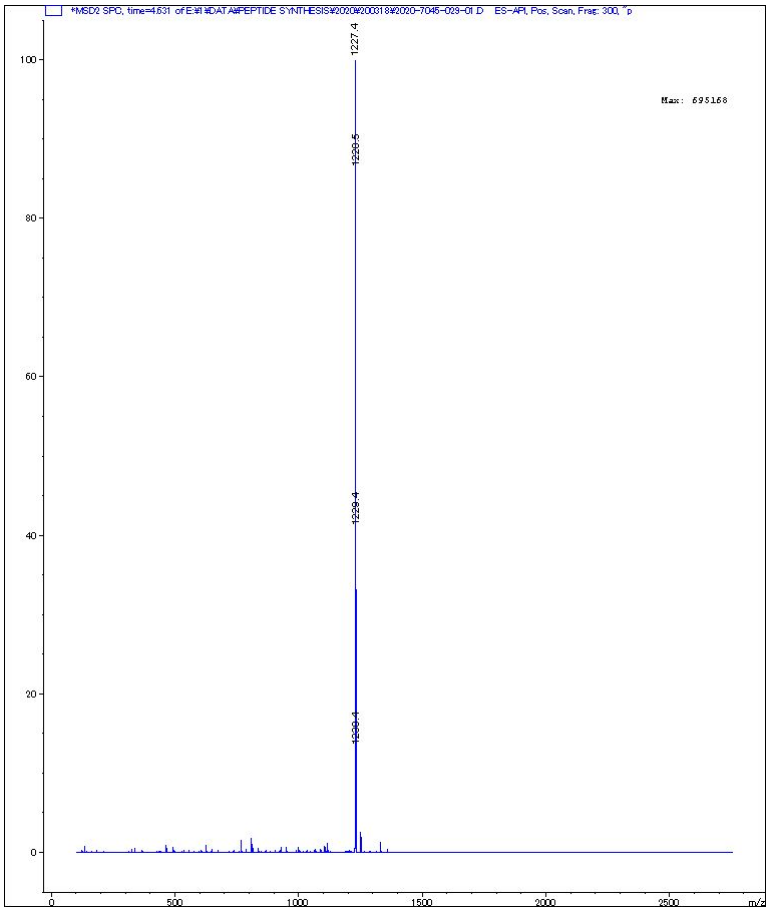


Peptide **15** was synthesized by Synthetic **Method S-C** with resin **19** (0.8 mol/g, 0.05 mmol, 65 mg). For amino acids next to α-methyl amino acid, double coupling was carried out for 4 min at 90 ℃. The protected linear peptide **20** was added CH_2Cl_2/TIPS/TFA (94/4/2, 100 mL/mmol) and shaken for 10 min at rt. After filtering the mixture, the residual resin was added CH_2Cl_2/TIPS/TFA (94/4/2, 100 mL/mmol) and shaken for 1 h at rt. After filtering the residual resin, the combined filtrate was added AcOEt and removed CH_2Cl_2 under reduced pressure. The solution was washed with $NaHCO_3$ and brine, dried over Na_2SO_4. After the solvent was removed under reduced pressure, the residue was dissolved in DMF (13 mL) and added E_3N (69 μL, 0.5 mmol). After being stirred overnight at rt, the cyclic peptide was purified using Purification **Method P-A**. The obtained pure fractions were combined and evaporated then added AcOEt. The solution was washed with sat. $NaHCO_3$ and brine, dried over Na_2SO_4. The solvent was removed under reduced pressure to give 15.3 mg (y. 21%) of peptide **15**. With Analytical **Method A-A**, UV purity (220 nm) = 82.7% (Gradient: retention time = 4.56 min): MS-ESI (m/z) calcd for $C_{66}H_{103}N_{10}O_{10}S$ $[M+H]^+$ 1227.5, found 1227.4.

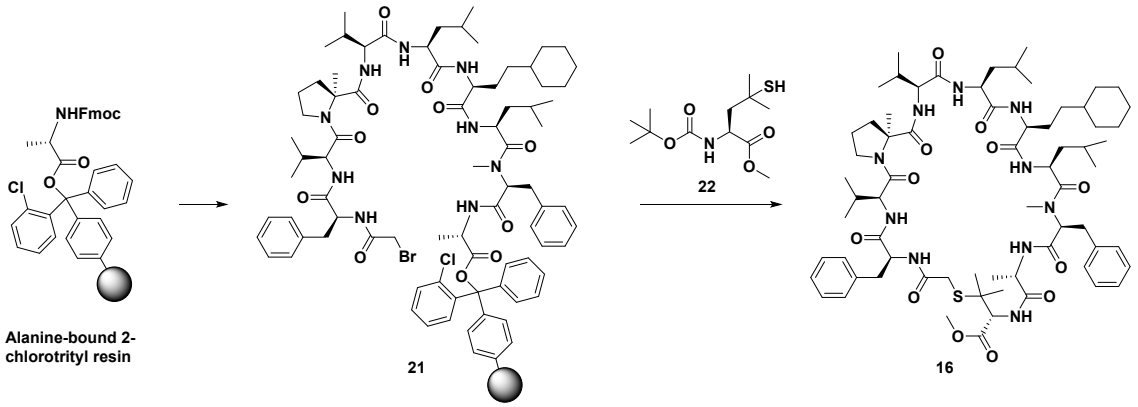


Signal 1: DAD1 A, Sig=220,4 Ref=off

Peak #	RT [min]	Type	Width [min]	Area %	Area
1	0.777	MM	0.264	4.505	756.090
2	4.556	MF	0.092	82.697	1.388e4
3	4.665	FM	0.054	5.002	839.594
4	5.793	MF	0.083	6.075	1.020e3
5	6.021	FM	0.098	1.721	288.881


+MSD2 SPC, time=4.631 of E:\#\DATA\PEPTIDE SYNTHESIS\2020\200318\2020-7045-029-01.D ES-API, Pos, Scan, Frag: 300, "p
Max: 695.168
1227.4
1228.5
1229.4
1230.1
100
80
60
40
20
0
0
500
1000
1500
2000
2500
m/z

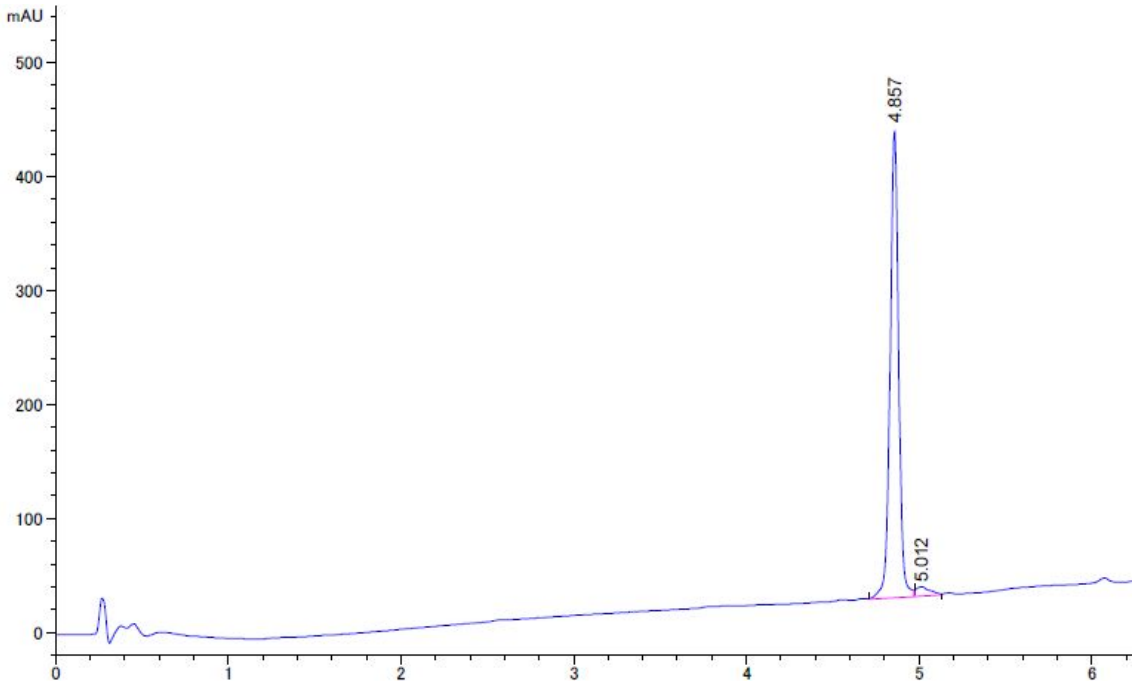
Synthesis of peptide **16**



Peptide **16** was synthesized by Synthetic **Method S-D** with alanine-bound 2-chlorotrityl resin (0.37 mol/g, 0.25 mmol, 676 mg). The coupling and deprotection protocol were repeated to afford the elongated linear peptide. After elongation with amino acids, to the resulting peptide-bound resin was added a solution of bromoacetic acid (5 equiv.), DIC (10 equiv.), Oxyma (5 equiv.) dissolved in DMF (40 mL/mmol). The mixture was agitated for 30 min at rt. After drained, The protected linear peptide **21** (0.43 mmol/g, 0.25 mmol, 576 mg) was obtained.

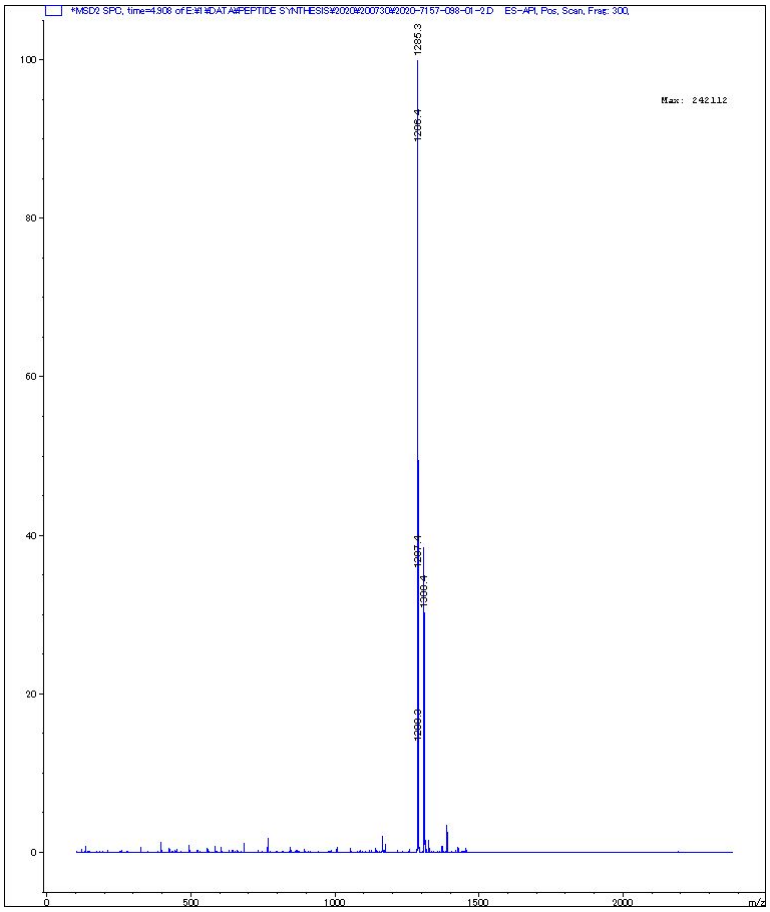
The protected linear peptide **21** (0.43 mmol/g, 0.05 mmol, 116 mg) was dissolved in DMSO (1 ml) and added **22** (69 mg, 0.25 mmol) and Et_3N (69 μL, 0.5 mmol). After the mixture was shaken overnight at rt, the mixture was drained and the resin was washed with CH_2Cl_2. The protected resin-bound peptide was added CH_2Cl_2/TFA/TIPS/H_2O (80/19/0.5/0.5, 20 mL/mmol) and shaken for 60 min at rt. After filtering the residual resin, the filtrate was quenched with sat. $NaHCO_3$ (20 mL) and washed with brine. The mixture was separated with the organic layer and the aqueous layer was extracted with AcOEt (30 mL). The combined organic layer was dried over Na_2SO_4 and removed under reduced pressure. The crude was remained Boc group. Therefore, the crude product was added TFA/CH_2CH_2 (1 mL/2 mL) and stirred for 10 min at rt twice to give the deprotected product as a colorless amorphous.

The deprotected product (0.05 mmol) was dissolved in DMF (10 ml) and added DIPEA (87 μL, 0.5 mmol), HATU (57 mg, 0.15 mmol). After being stirred for 7 h at rt, the mixture was added AcOEt and H2O. The mixture was separated with the organic layer and the aqueous layer was extracted with AcOEt. The combined organic layer was dried over Na_2SO_4 and removed under reduced pressure. The cyclic peptide was purified using Purification **Method P-B**. The obtained pure fractions were combined and evaporated then added AcOEt. The solution was washed with sat. $NaHCO_3$ and brine, dried over Na_2SO_4. After the solvent was removed under reduced pressure, the residue was triturated with MTBE/n-hexane to give 16 mg (y. 25%) of peptide **16** as a white powder. With Analytical **Method A-A**, UV purity (220 nm) = 96.8% (Gradient: retention time = 4.86 min): MS-ESI (m/z) calcd for $C_{68}H_{105}N_{10}O_{12}S$ $[M+H]^+$ 1285.7, found 1285.3.



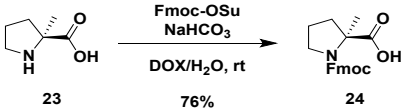
Signal 1: DAD1 A, Sig=220,4 Ref=360,100

Peak #	RT [min]	Type	Width [min]	Area %	Area
1	4.857	MF	0.058	96.808	1.431e3
2	5.012	MF	0.091	3.192	47.176


+MSD2 SPC, time=4.908 of E:\#HDATA#PEPTIDE SYNTHESIS#2020#20073O#2020-7157-088-01-2D ES-API, Pos, Scan, Frag: 300,
Max: 242112
1295.3
1296.4
1297.4
1298.4
1299.3
100
80
60
40
20
0
0
500
1000
1500
2000
m/z

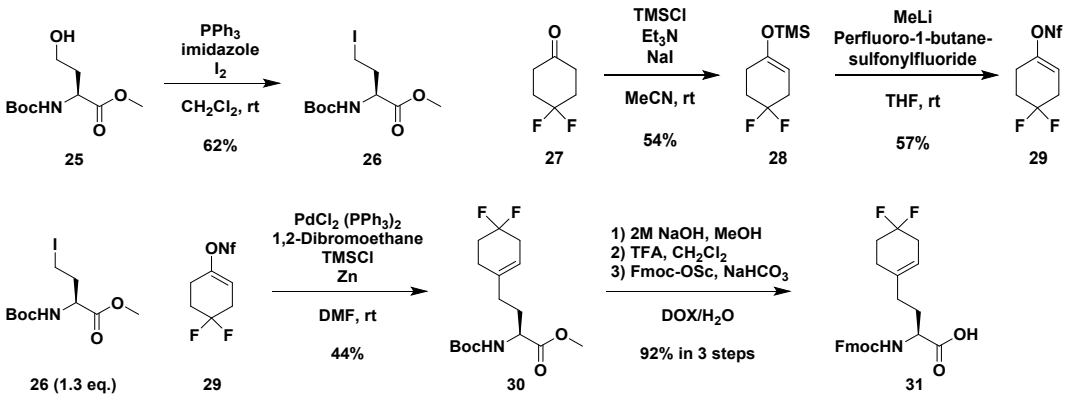
4. Synthesis of amino acids and resins

Synthesis of **24**



To a solution of **23** (10 g, 78 mmol) in DOX (100 mL) was added $NaHCO_3$ (32.8 g, 391 mmol) in H_2O (25 mL) and Fmoc-OSu (31.7 g, 94 mmol). After being stirred for 2 h at rt, the mixture was stored overnight. The mixture was neutralized with 2M HCl (pH = 4) and added AcOEt (200 ml). The mixture was separated with the organic layer and the aqueous layer was extracted with AcOEt (200 ml) again. The combined organic layer was washed with H_2O and brine, dried over Na_2SO_4. After the solvent was removed under reduced pressure, the crude was purified by flash column chromatography (0-50% $CHCl_3$:$CHCl_3$/MeOH/H_2O (10:1:0.1)). The obtained pure fractions were combined and evaporated, then crystallized with Et_2O/n-hexane to give **24** (20.8 g, 76%): ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 7.89 (2H, br m), 7.64 (2H, br m), 7.38 (4H, m), 4.26 (2H, s), 3.46 (2H, m), 2.09 (1H, m), 1.85 (3H, m), 1.42 (3H, s).

Synthesis of **31**



To a solution of PPh_3 (3.69 g, 14.1 mmol) and imidazole (0.96 g, 14.1 mmol) in CH_2Cl_2 (25 mL) was added iodine (3.57 g, 14.1 mmol). After being stirred for 30 min at 0 ℃, the mixture was added **25** (2.3 g, 9.37 mmol). After being stirred for 1 h at rt, the reaction mixture was quenched with sat. $Na_2S_2O_3$. The mixture was separated with the organic layer and the aqueous layer was extracted with CH_2Cl_2. The combined organic layer was removed under reduced pressure. The residue was added Ph_3PO and Et_2O followed by filtration to remove Ph_3PO. After the solvent was removed under reduced pressure, the crude was purified by flash column chromatography (0-60% AcOEt /Hexane) to give **26** (2.0 g, 62%) as a oil: ^{1}H-NMR (500 MHz, CDCl3) δ: 5.07 (1H, br m), 4.36 (1H, br m), 3.77 (3H, s),

3.18 (2H, t, J = 7.7 Hz), 2.41 (1H, br m), 2.18 (1H, br m), 1.45 (9H, s).

To a solution of **27** (10 g, 78 mmol) in DOX (100 mL) was added TMSCl (2.29 mL, 17.9 mmol), Et_3N (2.48 g, 17.9 mmol) and NaI (2.68 g, 17.9 mmol). After being stirred at rt overnight, the mixture was added n-hexane and quenched with 1M citric acid. The mixture was separated with the organic layer and the aqueous layer was extracted with Et_2O. The combined organic layer was removed under reduced pressure, the crude product **28** was used without further purification.

To a solution of **28** (1.2 g, 5.82 mmol) in THF (12 mL) was added MeLi (1.16 M solution: 6.02 mL, 6.98mmol) at 0 ℃. After being stirred overnight at rt, the mixture was added Perfluoro-1-butanesulfonyl fluoride (1.63 mL, 8.72 mmol) at 0 ℃. After being stirred overnight at rt, the mixture was added Et_2O and H_2O. The mixture was filtered and separated with the organic layer. The aqueous layer was extracted with Et_2O and the combined organic layer was removed under reduced pressure. The crude product was purified by flash column chromatography (0-10% AcOEt /Hexane) to give **29** (1.38 g, 57%): ^{1}H-NMR (500 MHz, CDCl3) δ: 5.69 (1H, s), 2.72 (2H, br m), 2.61 (2H, br m), 2.22 (2H, m).

This coupling reaction was performed by using the following papers (Tabor, M. G. and Shenvi, R. A.; Synthesis of Lepadiformine Using a Hydroamination Transform. *Org. Lett*. **2015**, *17*, 5776.)

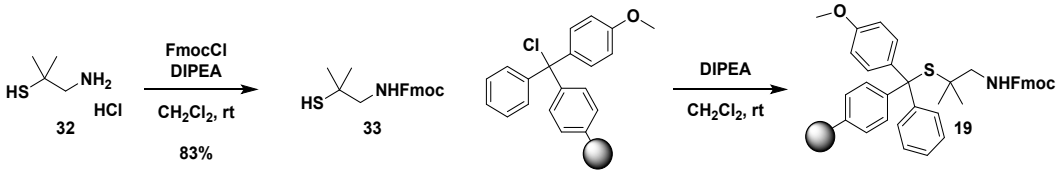
To a solution of Zinc (0.57 g, 8.74 mmol) and iodine (74 mg, 0.29 mmol) in DMF (3 mL) was degassed and replaced with N_2, stirred for 20 min at rt. The mixture was added **29** (1 g, 2.91 mmol) in DMF and degassed, replaced with N_2. After being stirred for 30 min at rt, the mixture was added **26** (1.38 g, 3.32 mmol) in DMF, bis(triphenylphosphine) palladium (II) Dichloride (0.21 g, 0.29 mmol) and degassed, replaced with N_2. After being stirred overnight at rt, the mixture was added AcOEt and filtered with a pad of celite. The filtrate was washed with brine and the aqueous layer was extracted with AcOEt. After the combined organic layer was removed under reduced pressure, the crude was purified by flash column chromatography (0-40% AcOEt /Hexane) to give **30** (0.7 g, 72%): ^{1}H-NMR (500 MHz, CDCl3) δ: 5.29 (1H, br s), 5.02 (1H, br s), 4.31 (1H, br s), 3.74 (3H, s), 2.49 (2H, br m), 2.21 (2H, br m), 2.14 (5H, m), 1.75 (1H, m), 1.45 (9H, s).

To a solution of **30** (267 mg, 0.8 mmol) in MeOH (5.3 mL) was added 2M NaOH (4 mL, 8 mmol). After being stirred overnight at rt, the mixture was neutralized with 1M citric acid. The mixture was separated with the organic layer and washed with H_2O. After the separated organic layer was removed under reduced pressure, the crude product was used without further purification.

To a solution of crude product (255 mg, 0.8 mmol) in CH_2Cl_2 (3 mL) was added TFA (3 mL, 38.9 mmol). After being stirred for 1 h at rt, the solvent was removed under reduced pressure and the crude product was used without further purification.

To a solution of crude product (266 mg, 0.8 mmol) in DOX (4 mL) was added $NaHCO_3$ (671 mg, 8 mmol) in H_2O (2 mL) and Fmoc-OSu (350 mg, 1.3 mmol). After being stirred for 4 h at rt, the mixture was neutralized with 1M citric acid and AcOEt. The mixture was separated with the organic layer and

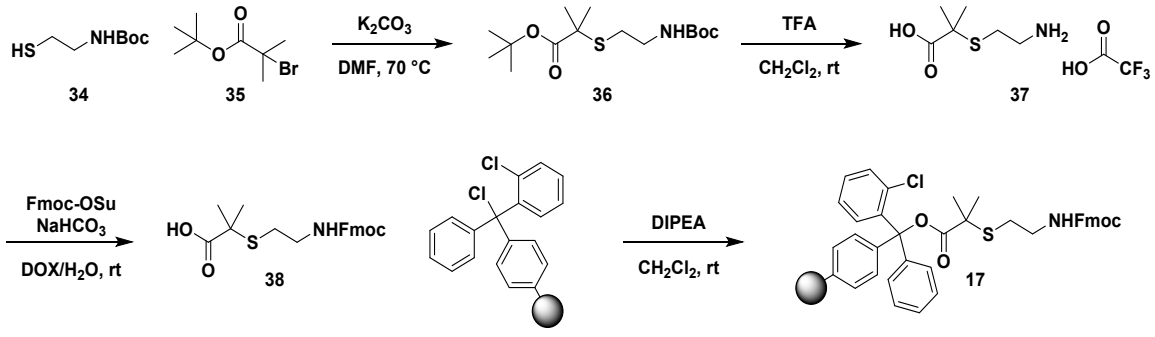
removed under reduced pressure. The crude was purified by flash column chromatography (0-20% CHCl3:CHCl2/MeOH/H_2O (10:1:0.1) to give **31** (323 mg, 92% in 3 steps): MS-ESI (m/z) calcd for C25H26F2NO4 $[M+H]^+$ 442.2, found 442.2.Synthesis of resin **19**



To a solution of **32** (2.74 g, 19.3 mmol) in CH_2Cl_2 (60 mL) was added DIPEA (6.76 mL, 38.7 mmol) and FmocCl (4.75 g, 18.4 mmol) in CH_2Cl_2 (15 mL) at 0 ℃. After being stirred for 30 min at rt, the mixture was poured into 0.2 M HCl/AcOEt followed by separation of the layers. The aqueous layer was extracted with AcOEt, and the combined organic layer was washed with H_2O and brine, dried over Na_2SO_4. After the solvent was removed under reduced pressure, the crude was purified by flash column chromatography (5-25%, AcOEt/hexane) to give **33** (5.25 g, 83%) as a pale yellow oil: ^{1}H-NMR (500 MHz, CDCl3) δ: 7.77 (2H, d, J = 7.5 Hz), 7.61 (2H, d, J = 7.4 Hz), 7.41 (2H, t, J = 7.5 Hz), 7.32 (2H, m), 5.22 (1H, br s), 4.44 (2H, d, J = 6.9 Hz), 4.24 (1H, t, J = 6.8 Hz), 3.28 (2H, d, J = 6.4 Hz), 1.35 (6H, s).

4-Methoxytrityl chloride resin (1.8 mmol/g, 3.73 g, 6.72 mmol) was added **33** (2.00 g, 6.11 mmol) in CH_2Cl_2 (30 mL) and DIPEA (4.27 mL, 24.4 mmol). After being shaken for 5 h at rt, the mixture was MeOH (3 mL). The reaction mixture was shaken for 1 h at rt and drained. After the resin was added CH_2Cl_2/MeOH/DIPEA (17:2:1, 6.5 mL/mmol), the mixture was shaken for 1 h at rt and drained. The remained resin was washed with DMF, CH_2Cl_2, and MTBE to give **19** (1.2 mmol/g, 5.1 g) as a yellow sand.

Synthesis of resin **17**



To a solution of **34** (2.68 g, 12 mmol) and **35** (2.13 g, 12 mmol) in DMF (26.8 mL) was added K_2CO_3 (1.66 g, 12 mmol) at rt. After being stirred for 5 h at 70 ℃, the mixture was quenched with water and the aqueous layer was extracted with Et_2O. The mixture was separated with the organic layer and dried

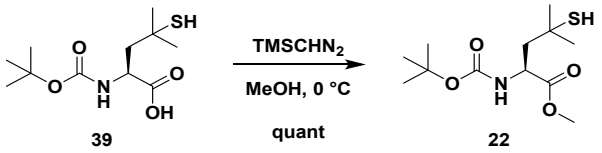
over Na_2SO_4. After the solvent was removed under reduced pressure, the crude **36** was used without further purification.

To a solution of crude **36** in CH_2Cl_2 (38 mL) was added TFA (37.6 mL) and triisopropylsilane (0.4 mL) at rt. After being stirred for 40 min at rt, the solution was removed under reduced pressure and the crude **37** was used without further purification.

To a solution of crude **37** in DOX (66 mL) was added $NaHCO_3$ (10.1 g, 120 mmol) H_2O (33 mL) and Fmoc-OSu (4.85 g, 14.4 mmol) at rt. After being stirred for 1 h at rt, the mixture was added NaHCO3 (7.06 g, 84 mmol) at rt. After being stirred for 1 h at rt, the mixture was added Fmoc-OSu (2.43 g, 7.2 mmol) and NaHCO3 (7.06 g, 84 mmol) at rt. After being stirred for 1 h at rt, the mixture was stored overnight. The mixture was neutralized with 2M HCl and the aqueous layer was extracted with AcOEt. The mixture was separated with the organic layer and dried over Na_2SO_4. After the solvent was removed under reduced pressure, the crude was purified by flash column chromatography (0-50% EtOAc/Hexane) to give **38** (5.66 g) as a mixture of 9-Methylenefluorene: MS-ESI (m/z) calcd for C21H24NO4S $[M+H]^+$ 386.1, found 386.0

2-Chlorotrityl chloride resin (8.04 g, 13.4 mmol) was added **38** (5.66 g) in CH_2Cl_2 (60 mL) and DIPEA (10.4 mL, 59.5 mmol). After being shaked overnight at rt, the mixture was added 2-Chlorotrityl chloride resin (2.15 g, 3.33 mmol) at rt. After being shaked for 3 h at rt, the mixture was removed the solution and the resin was added CH_2Cl_2/MeOH/DIPEA (17:2:1, 3 mL/mmol). After being shaked for 1 h at rt, the mixture was removed the solution and the resin was added CH_2Cl_2/MeOH/DIPEA (17:2:1, 3 mL/mmol) again. After being shaked for 20 min at rt, the mixture was removed the solution and the resin was washed DMF and CH_2Cl_2, dried to give **17** (0.56 mmol/g, 13.38 g).

Synthesis of **22**



To a solution of **39** (0.5 g, 1.9 mmol) in MeOH (5 mL) was added $TMSCH_2$ (0.6M solution: 9.49 mL, 5.7 mmol) at 0 ℃. After being stirred for 30 min at 0 ℃, the mixture was quenched AcOH, then sat. $NaHCO_3$ (30 mL). The aqueous layer was extracted with AcOEt (30 mL x2) twice, the combined organic layer was washed with H_2O and brine, dried over Na_2SO_4. The solvent was removed under reduced pressure to give **22** (0.56 g, quant) as a colorless amorphous: ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 7.17-7.14 (1H, m), 4.20-4.15 (1H, m), 3.63 (3H, s), 3.36 (1H, dd, *J* = 11.2, 4.4 Hz), 1.38 (9H, s), 1.35 (5H, s).

5. Biological Assays

HIV-1 Protease Preparation for Biological Assay

HIV-1 protease was expressed in Escherichia coli BL21 (DE3) pLysS transformed with plasmid pET3b-PR-WT. (Cihlar, T.et al., Suppression of HIV-1 Protease Inhibitor Resistance by Phosphonate-mediated Solvent Anchoring. *J. Mol. Biol.* **2006**, *363*, 635−647) The expressed enzyme was isolated and refolded as described below. Bacterial cell pellets were re-suspended in 10 mM Tris−HCl (pH 8.0) buffer and centrifuged at 10,000g for 10 min. Cell pellets were re-suspended in suspension buffer, 10 mM Tris−HCl (pH 8.0), 2 mM EDTA, 1 mM PMSF, and 50 mg/100 mL lysozyme chloride. 0.1% NP-40 was added to cell pellets and stirred for 10 min. 10 mM MgCl2, 4 U DNase solution was added to the sample and cell pellets were homogenized physically and centrifuged. The inclusion bodies were washed with a suspension buffer containing Triton X-100 (1% v/v) two times. HIV-1 protease was solubilized in a solubilization buffer, pH 7.5, containing 25 mM Tris, 1 mM EDTA, 10 mM DTT, and 8 M urea by stirring for 120 min at 5°C. The sample was centrifuged at 10,000 g for 60 min. The supernatant was filtered through a 0.45 microfilter and loaded onto an anion exchange column equilibrated with a solubilization buffer. HIV-1 protease was refolded by 15-fold dilution into 10 mM sodium acetate (pH 4.5) and 3 mM DTT at 25°C. The pH was raised to 6.0 with 1 M NaOH, and the sample was rapidly cooled to 4°C. The sample was loaded onto a cation exchange column equilibrated with 10 mM sodium acetate (pH 6.0), 10% (v/v) glycerol, and 1 mM DTT buffer. Finally, the sample was purified by gel filtration chromatography.

Fluorogenic Peptide Cleavage Assay

The fluorogenic substrate [EDANS-RESGIFLETSKR-DABCYL] (Windsor, I. W. et al., Fluorogenic Assay for Inhibitors of HIV-1 Protease with Sub-picomolar Affinity. *Sci. Rep*. **2015**, *5*, 11286) was purchased from PEPTIDE INSTITUTE, INC. Assays were performed in an assay buffer (50 mM sodium acetate buffer, pH 5.0, containing 0.1 M NaCl, 2% v/v DMF, 0.002% v/v TFA and 0.025% v/v Tween 20) and conducted at room temperature. HIV-1 protease (final concentration: 50 pM) was incubated with compounds for 15 min. Reactions were initiated by the addition of the substrate (final concentration: 2 μM) and incubated for 60 min. The fluorescence was measured at 485 nm with an excitation wavelength of 340 nm using a microplate reader. The 50% inhibitory concentration (IC_{50}) of substrate cleavage by HIV-1 protease was determined from concentration response curves using a four-parameter logistic curve fitting model. The IC_{50} of Darunavir was 0.037 nM. The experiments were performed once or in duplicate, and the data were presented as the single or the arithmetic mean.

Cells and Virus

Human T cell line MT-4 cells were obtained from Dr. Koyanagi (Institute for Virus Research, Kyoto University), and Molt-4 cells persistently infected with the HIV-1 IIIB strain were obtained from Dr.

Harada (Kumamoto University). These cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 μg/mL kanamycin sulfate. The cell culture supernatant of Molt-4/IIIB was used as an HIV-1 IIIB solution.

Anti-HIV-1 Activity Assay in MT-4 Cells

Antiviral activity was measured using MT-4 cells, as described previously. (Kobayashi, M.et al., In Vitro Antiretroviral Properties of S/GSK1349572, a Next-Generation HIV Integrase Inhibitor. *Antimicrob. Agents Chemother*. **2011**, *55*, 813−821*)* MT-4 cells (2.5×10^4 cells) in 96-well plates were incubated with compounds at 37°C, 5% CO2 for 1 h. After incubation, the MT-4 cells were infected with HIV-1 IIIB at a 50% tissue culture infectious dose of 4 to 10 and cultured at 37°C, 5% CO_2 for 4 days. At the end of the culture period, the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] solution was added to MT-4 cells and incubated at 37°C, 5% CO_2 for 1 h. After incubation, MT-4 cells were lysed, followed by mixing to allow for color development. Absorbance was read at 560 nm (subtract background at 690 nm) using a microplate reader. The effective concentration achieving 50% inhibition (EC_{50}) of cell death caused by HIV-1 infection was determined from concentration−response curves using a four-parameter logistic curve fitting model. The EC_{50} of Darunavir was 2.8 nM. The experiments were performed once or in duplicate, and the data were presented as the single or the arithmetic mean.

6. Pharmacokinetic studies

Metabolic stability

All studies with human samples were approved by the Ethics Committee on Human Tissue and Genome Research at Shionogi & Co., Ltd. (Toyonaka, Japan). Using commercially available pooled human hepatic microsomes and S9, which is the supernatant fraction obtained from the liver homogenate by centrifuging, rat hepatic microsomes and S9 by preparation of in-house, a test compound was reacted for a constant time, a remaining rate was calculated by comparing a reacted sample and an unreacted sample, thereby, a degree of metabolic stability was assessed.

A reaction was performed (oxidative reaction) at 37°C for 0 minute or 30 minutes in the presence of 1 mmol/L NADPH in 0.2 ml of a buffer (50 mmol/L tris-HCI pH 7.4, 150 mmol/L potassium chloride, 10 mmol/L magnesium chloride) containing 0.5 mg protein/ml of human or rat liver microsomes. When using liver S9, A reaction was performed at 37°C for 0 minute or 60 minutes in 0.2 ml of a buffer (50 mmol/L tris-HCI pH 7.4, 150 mmol/L potassium chloride, 10 mmol/L magnesium chloride) without NADPH containing 0.5 mg protein/ml of human or rat liver S9. After the reaction, 50 μL of the reaction solution was added to 100 μL of a methanol/acetonitrile = 1/1 (v/v), mixed and centrifuged at 3000 rpm for 15 minutes. The test compound in the supernatant was quantified by LC-MS/MS, and a remaining amount of the test compound after the reaction was calculated, letting a compound amount at 0 minute reaction time to be 100%. The experiments were performed in duplicate, and the data were presented as the arithmetic mean.

Solubility

Solubility of the compound was determined under the condition of 0.5% DMSO. Compounds were dissolved in DMSO to give a 10 mM DMSO solution. The DMSO stock solution was diluted with Japanese pharmacopoeia (JP) 1 (pH 1.2), JP2 (pH 6.8), JP2 (pH 6.8) containing 50 mM taurocholic acid so that 0.5% of the final DMSO concentration was added. The solution was incubated for 1 hours at 37 °C and filtrated. The filtrate was diluted by MeOH/H2O (1/1) and was analyzed by HPLC or UHPLC/MS. The experiments were performed in duplicate, and the data were presented as the arithmetic mean.

Serum protein binding

The unbound fraction of the present compound in serum is measured using rat serum. The reactive conditions are as follows: Evaluation method, Equilibrium dialysis; Reaction time, 24 hours; Reaction temperature, 37°C; Concentration of the present compound, 2 μg/mL. The test solution is added to each serum and the mixture is agitated to prepare the serum samples at the concentration mentioned above. Each serum sample is added into one side of the cell and phosphate buffered saline (PBS) is added into the other side to perform equilibrium dialysis at 37°C for 24 hours. Then, the concentration

of the compounds in the samples that are obtained from both sides was measured by LC-MS/MS. The ratio (%) of PBS concentration to serum concentration is expressed as unbound fraction. The experiments were performed in duplicate, and the data were presented as the arithmetic mean.

EPSA

Exposed polar surface-area (EPSA) analysis by supercritical fluid chromatography (SFC) EPSA analysis was performed by using a previously reported procedure with modifications (Goetz GH et al., EPSA: a novel supercritical fluid chromatography technique enabling the design of permeable cyclic peptides. *ACS Med. Chem. Lett.*, **2014**, *5*, 1167-1172., Goetz GH et al., High throughput method for the indirect detection of intramolecular hydrogen bonding. *J Med. Chem*., **2014**, *57*, 2920-2929.). The SFC instrument used in this experiment was a JASCO supercritical fluid chromatography system. Ammonium formate diluted to 20 mmol/L in HPLC grade methanol was used as the mobile-phase modifier. Analysis was performed using a 4.6 mm x 250 mm Sumichiral OA-4100 column (Sumika Chemical Analysis Service, Osaka, Japan) with 5-mm particles at 40°C. The flow rate was set at 2 mL/min with the outlet backpressure set to 150 bar. The mobile-phase composition varied from 5 to 50% modifier at 2%/m1 in a linear gradient, holding at 50% for 2.5 min, and reverting to the original 5% until the end of the run. Antipyrine, pindolol, diclofenac sodium salt, and m-nitrobenzoic acid were used as calibration standards. EPSA was calculated from the following Equation based on the calibration:

$$EPSA = 5.686 \times SFC\ tR + 22.718$$

Each individual compound was dissolved to approximately 3 mmol/L in DMSO and methanol, and 5 μL of the solution was injected into the SFC instrument. The experiments were performed once.

Rat in vivo study

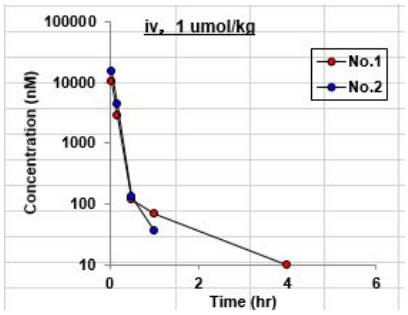
Animal care and all experimental procedures were performed with the approval of the Institutional Animal Care and Use Committee of Shionogi in terms of the 3R (Replacement/Reduction/Refinement) principle. The study in male Sprague-Dawley rats (8 weeks old) was conducted at Shionogi & Co., Ltd. The rats were allowed free access to tap water and solid laboratory food (CE-2, CLEA Japan, Inc.) throughout the acclimation and experimental periods. Briefly, after a single intravenous administration of the compounds dissolved in mixtures of DMSO and propylene glycol at 1 μmol/kg to rats (n=2), blood samples (0.2 mL) were collected using anticoagulants-containing syringe at 0.05, 0.17, 0.5,1, 2, 4, and 6 h. After a single oral administration of the compounds suspended in mixtures of DMSO and 0.5% methyl cellulose aqueous solution at 2 μmol/kg to rats (n=2), blood samples (0.2 mL) were collected using anticoagulants -containing syringe at 0.5, 1, 2, 4, 8 and 24 h. The blood samples were centrifuged, and the plasma samples were obtained. The compound concentration in plasma was measured by LC-MS/MS. The data were presented as the arithmetic mean.

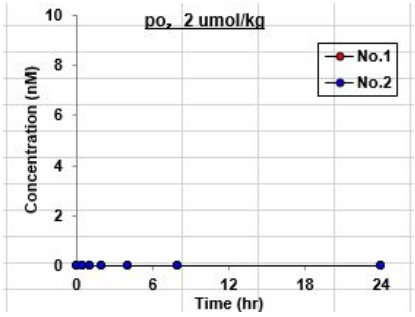
PK analysis of the plasma concentration was performed using the expressed figures with Phoenix WinNonlin® 6.4 (Certara, Princeton, NJ) based on a noncompartmental model with uniform weighting. The area under the plasma concentration from time zero to infinity (AUC) were calculated using the trapezoidal method. CLtot was obtained by dose/AUC. The bioavailability (BA) was calculated from the AUC of the oral administration group and intravenous administration group. The mean value of PK parameters was represented.

7. Rat pharmacokinetic data of peptide 2, 11, 15, 16 after IV and PO dosing

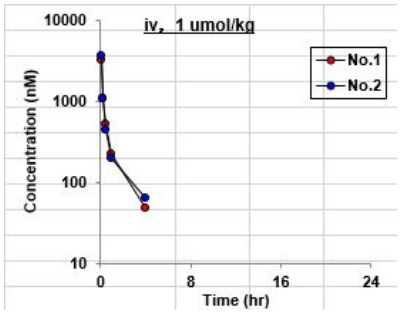
Rat plasma concentrations following IV administration (1 μmol/kg) and PO administration (2 μmol/kg). The lower limit of quantification (LLOQ) of each compound were 10 nM.

Peptide 2

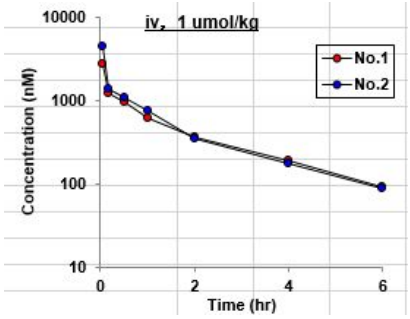


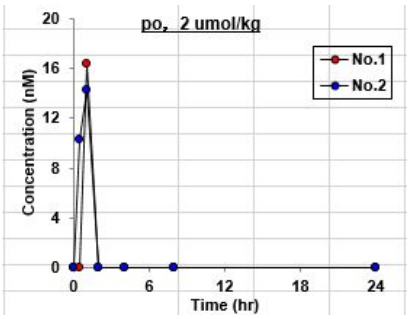


Peptide 11 (po: not detected)

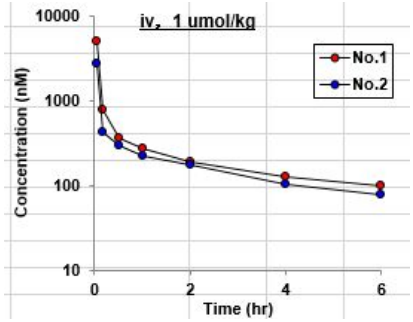


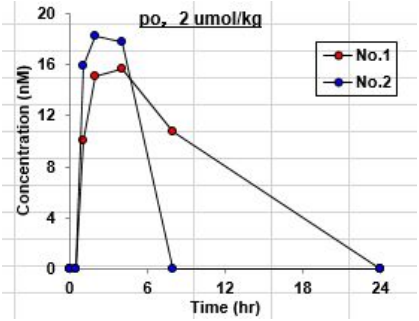
Peptide 15





Peptide 16





8. X-ray crystal structure analysis

Protein Expression and Purification for Crystallization.

The DNA of HIV-1 protease was cloned into pET3b and expressed in Escherichia coli BL21 (DE3) pLysS. Protein expression was carried out as described.30 Cells were lysed by sonication in a lysis buffer (10 mM Tris pH 8.0, 2 mM ETDA, 1 mM PMSF, 30 μL of Lysonase Bioprocessing Reagent (Merck)). The inclusion bodies were washed with the lysis buffer and solubilized in 25 mM Tris pH 7.5, 1 mM EDTA, 10 mM DTT, and 8 M urea. After centrifugation, the supernatant was diluted with 10 mM CH_3COONa, pH 4.5 and 3 mM DTT. The pH was raised to 6.0 with 1 M NaOH, and the sample was rapidly cooled to 4 °C. After centrifugation, the supernatant was filtered through a 0.22 mm filter and loaded onto a column of HiTrap SP (cytiva) pre-equilibrated with 10 mM CH_3COONa pH 6.0, 1 mM DTT. Bound protein was eluted with 0.5 M NaCl in 10 mM CH_3COONa, pH 6.0 and 1 mM DTT. The eluted protein was further purified by Superdex75 previously equilibrated with 10 mM CH_3COONa, pH 5.0 and 1 mM DTT.

X-ray Crystallography.

The HIV-1 protease inhibitor complexes were prepared by mixing the protein with a five-fold molar excess of inhibitors (in DMSO) and concentrated by ultrafiltration to a protein concentration of 5 mg/mL. Crystallization was performed by the sitting-drop vapor diffusion method at 20 °C. The crystallization drops consisted of 0.3 μL of HIV-1 protease inhibitor complex and 0.3 μL of reservoir solution. The reservoir solution for the HIV-1 protease compound 1 complex contained contained 22.5% w/v polyethylene glycol 3,350 and 0.1 M magnesium chloride hexahydrate. For diffraction measurement at 100 K, crystals were soaked in the reservoir solution supplemented with 20% glycerol and cryocooled in liquid nitrogen. X-ray diffraction data were collected on a RAXIS IV++ (Rigaku). Diffraction data were processed using HKL-2000 (Otwinowski, Z.; Minor, W. Processing of X-ray Diffraction Data Collected in Oscillation Mode. *Methods Enzymol*. **1997**, *276*, 307– 326). Structures were solved by molecular replacement using protease from the PDB structure (PDB ID: 4LL3) (Kozisek, M.; Lepsik, M.; Saskova, K. G.; Brynda, J.; Konvalinka, J.; Rezacova, P. Thermodynamic and Structural Analysis of HIV Protease Resistance to Darunavir - Analysis of Heavily Mutated Patient-Derived HIV-1 Proteases. *FEBS J*. **2014**, *281*, 1834–1847) as a search model. Structure refinement and manual model rebuilding were performed with Refmac535 and COOT,36 following accession codes:7YF6 (PDB code). X-ray data collection and refinement statistics for compounds are described in the Supporting Information (Table S1).

Table S-1

X-ray data collection and refinement statistics for compound 1 are described the below table.

PDB code	7YF6
Data collection statistics	
Space group	$P2_12_12_1$
Cell parameters (Å)	31.877 49.325 122.078
Wavelength (Å)	1.5418
Resolution (Å)[a]	31.39 - 2.01 (2.082 - 2.01)
Number of unique reflections[a]	13466 (1303)
Multiplicity[a]	7.3 (6.7)
Completeness (%)[a]	99.91 (99.69)
R_{merge} (%)[a,b]	6.69 (28.72)
CC1/2[a]	0.998 (0.958)
Mean I/σ(I)[a]	12.9 (6.4)
Refinement statistics	
Resolution range (Å)	31.39 - 2.01
Number of reflections in working set[a]	13460 (1303)
Number of reflections in test set[a]	635 (60)
R_{work} (%)[a, c]	19.56 (22.97)
R_{free} (%)[a, d]	23.74 (26.63)
Rmsd bond length (Å^2)	0.006
Rmsd angle (°)	1.18
Number of water molecules in AU	98
Average *B* factor (Å^2)	24.87
Ramachandran plot statistics	
Residues in favored/allowed regions (%)	99/1

[a] Values in parentheses are for the highest resolution shell.

[b] $R_{merge} = \Sigma| I - \langle I \rangle |/\Sigma I$, where I is the intensity of observation I and $\langle I \rangle$ is the mean intensity of the reflection.

[c] $R_{work} = \Sigma||F_o| - |F_c||/\Sigma|F_c|$, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively.

[d] R_{free} was calculated using a randomly selected 5% of the data set that was omitted through all stages of refinement.

9. mRNA Display selection procedure

The DNA of HIV-1 protease was cloned into pET3b and expressed in Escherichia coli BL21 (DE3) pLysS. HIV-1 protease was biotinylated using EZ-Link Maleimide-PEG2-Biotin (Thermo Fisher Scientific) at molar ratios of 1:5. About 100% of the HIV-1 protease was labeled with biotin by analyzing the molecular weight using an ultraflex II MALDI-TOF-MS (Bruker Corporation).

A selection campaign to discover macrocyclic peptides that bind to HIV-1 protease was performed using an in vitro translated peptide library, which was produced through Flexizyme-mediated genetic code reprogramming as previously described (Goto Y, Katoh T, Suga H. Flexizymes for genetic code reprogramming. *Nat. Protocols* **2011**, *6*, 779-790, Kashiwagi K and Reid C. P. Rapid display method in translational synthesis of peptide. WO2011049157, Ishizawa T, Kawakami T, Reid P.C, Murakami H. TRAP display: a high-speed selection method for the generation of functional polypeptides. *J. Am. Chem. Soc*. **2013**, *135*, 5433-5440). Briefly, L-Met was replaced with N-chloroacetyl-L-Phe as the initiator amino acid. Elongator amino acids were composed of the natural amino acids Gly, Ser, Asn, Asp, Arg, Val, Leu, Pro, Tyr, Trp and Cys, as well as the non-natural amino acids MePhe, MeGly, MeNle, and MeAla. All macrocyclic peptides in this library were composed of 10 to 14 amino acids, including the fixed C-terminal L-cysteine which cyclizes on the N-terminal chloroacetyl. From the initial library consisting of $>10^{13}$ unique peptides, HIV protease-specific binders were enriched using biotinylated HIV protease-bound streptavidin conjugated magnetic beads. Non-specific binders were removed using streptavidin conjugated magnetic beads only. Library HIV protease binding steps were performed in an iterative manner. Binding stringency was increased in latter rounds through temperature, incubation times, and number of wash steps. Selection rounds were used for next generation sequencing to analyze the peptide sequences that were enriched.