Supporting Information

Facile Synthesis of Macrocyclic Peptide Toxins of GpTx-1 and its Analogue

Chao Chen,*[a] Mei Hong,*[a] Xiaoqi Guo,[b,c] Fangming Wu,[c] Changlin Tian,*[b,c] Yangding Wang and Zhaoqing, Xu*[d]

[a] School of Chemical Biology & Biotechnology, Peking University Shenzhen Graduate School, Shenzhen 518055, China.

[b] School of Life Sciences, University of Science and Technology of China, Hefei 230027, China

[c] High Magnetic Field Laboratory, Chinese Academy of Sciences, Hefei 230031, China

[d] Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Science, Lanzhou University. 199 West Donggang Road, Lanzhou 730000, China

TABLE OF CONTENTS

LIST OF FIGURES

General information

1.1 Materials

N^{α}-Fmoc protected amino acids were purchased from CD CHEMPEP (*Chengdu*, *China*), or ChengDu ChengNuo New-Tech Co., Ltd (*Chengdu*, *China*). Rink Amide AM resin was purchased from XIAN SUNRESIN (Xian, China). HPLC-quality acetonitrile (*ACN*) was purchased from *Shanghai Xingke.*

The following compounds and reagents were purchased from China local suppliers: *PyBop* (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate), piperidine (*PIP*), *DIC* (*N,N*'-diisopropylcarbodiimide), trifluoroacetic acid (*TFA*), *N,N*-diisopropylethylamine (*DIEA*), *3,6*-dioxa-*1,8*-octanedithiol (*DODT*), triisopropylsilane (*TIS*), oxidized glutathione, and reduced glutathione, Tri(hydroxymethyl) aminoethane) (*Tris*), dichloromethane (*DCM*), methanol (*MeOH*), *N,N*-dimethylforamide (*DMF*), methyl *tert*-butyl ether (*MTBE*).

1.2 Structure of GpTx-1

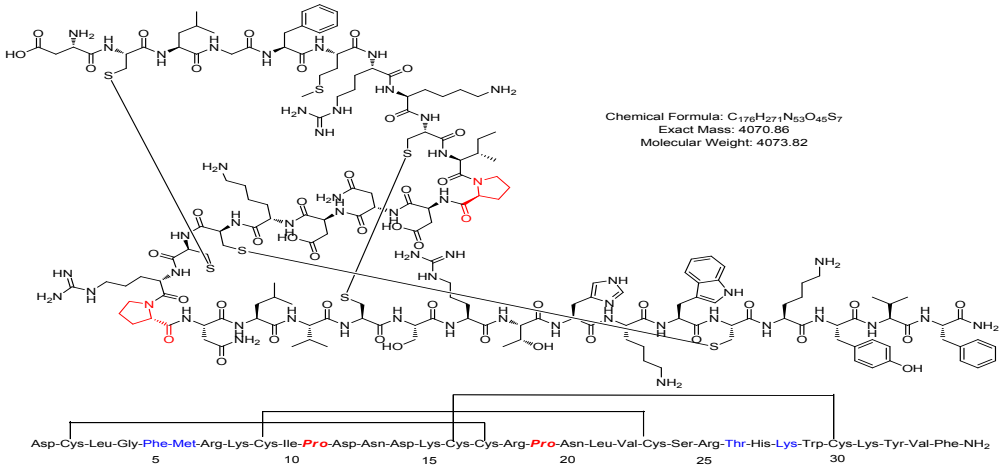


Figure S1. The chemical structure and sequence of GpTx-1

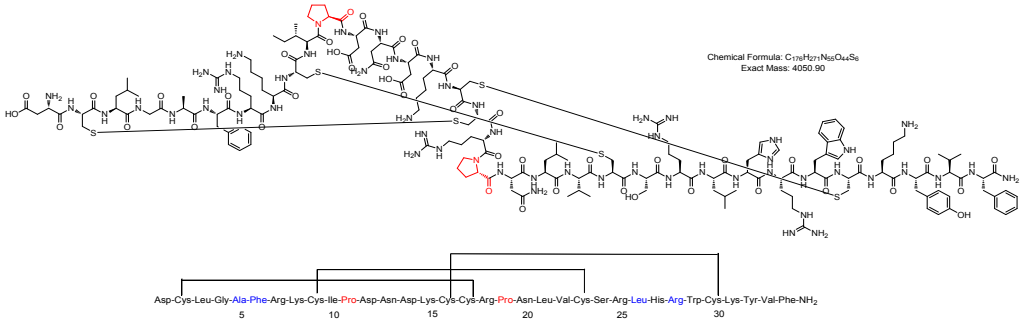


Figure S2. The chemical structure and sequence of [Ala^{5},Phe^{6},Leu^{26},Arg^{28}] GpTx-1

(GpTx-1-71)

1.3 Characterization methods

Analytical RP-HPLC was performed at 35 ^{o}C for column temperature on the Shimadzu LC 20 with UV detector SPD-20A or Waters 2695 with UV detector 2487 using Inertsil ODS-SP column (4.6 x 15 mm, 5 μm, 100 Å) or Waters X-Bridge ((4.6 x 15 mm, 5 μm, 100 Å). The RP-HPLC gradient was started at 10% of mobile phase B (0.1% TFA in CH_3CN), then increased to 65% of mobile phase B over 20 or 30 min (A: 0.1% TFA in H_2O). For full protected peptide segments, the HPLC gradient of Mobile phase A was increased from 70% to 95% over 20 minutes.

Semi-preparative RP-HPLC was performed on the ULTIMAT 3000 Instrument (DIONEX). UV absorbance was measured using a photodiode array detector at 215 and 254 nm. The RP-HPLC gradient was started at 10% of B (0.1% TFA in CH_3CN), then increased to 65% of B over 60 min (A: 0.1% TFA in water). The flow rate was 0.8 mL/min.

Mass spectra were acquired at LCMS-8030 (Shimadzu) or ABI Q-star Elite (High resolution).

2.0 Experimental procedure

2.1 Peptide Synthesis

Segments of GpTx-1, analogue #71 were synthesized using N^{α}-Fmoc solid-phase peptide synthesis (SPPS) methodologies with appropriate orthogonal protection and supporting matrix strategies. The following side chain protection strategies were employed for standard amino acid residues: Asn(Trt), Tyr(^{t}Bu), Cys(Trt), Lys(N^{ϵ}-Boc), Trp(Boc), His(Trt), Thr(^{t}Bu), Asp(O^{t}Bu), Ser(^{t}Bu), Arg(Pbf), Tyr(^{t}Bu), Tyr(^{t}Bu), Cys(Trt), Gln(Trt). The two linear peptide segments (H-[20-34]-AM resin of GpTx-1 and Analogue 71) were synthesized on a 3 mmol scale using AM resin (100−200 mesh, 1% DVB, 1.0-1.2 mmol/g initial loading (Xian SunResin, Xi'an China). The segment Fmoc-[1-11]-CTC or Boc-[1-11]-CTC and Fmoc-[12-19]-CTC were synthesized on a 2 mmol scale using 2-CTC resin with 0.9-1.2 mmol/g initial loading (Xian SunResin, Xi'an China).

2.2 Peptide Synthesis by stepwise strategy

At the beginning, a standard stepwise strategy was employed to organize the tartgeting peptides according the literature (*J. Med. Chem.* 2015, 58, 2299−2314.). The crude GpTx-1 was acquired with 41.1% HPLC purity (Figure S4). The prufied GpTx-1 was obtained by RP-HPLC with 98.0% HPLC purity in 2.5% yield based on the crude linear peptide (*proline-based segement strategy*: 7.5% yield).

When its analogue GpTx-1-71 was assembled by stepwise solid phase peptide synthesis, the linear peptide of GpTx-1-71 was obtained with <40% HPLC purity (Figure S3). After oxidative folding, GpTx-1-71 was generated with a relative complicated HPLC profile

(Figure S4) and it could not be easy to acquire higher than 98% HPLC purity in <1% yield after purification (*proline-based segement strategy*: 3.5% yield).

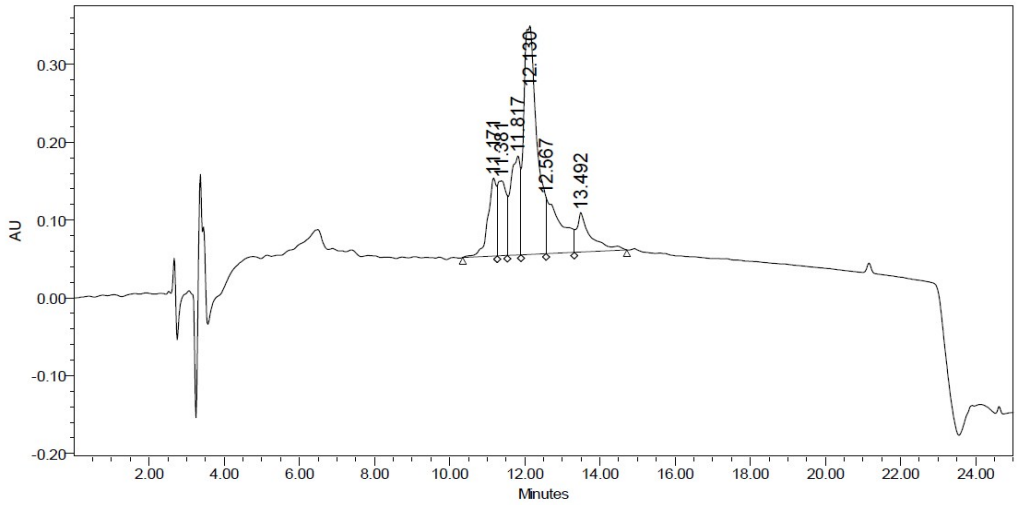


Figure S3. HPLC profile of linear GpTx-1 synthesized by standard stepwise strategy (HPLC Gradients: 10-65% for 20 mins)

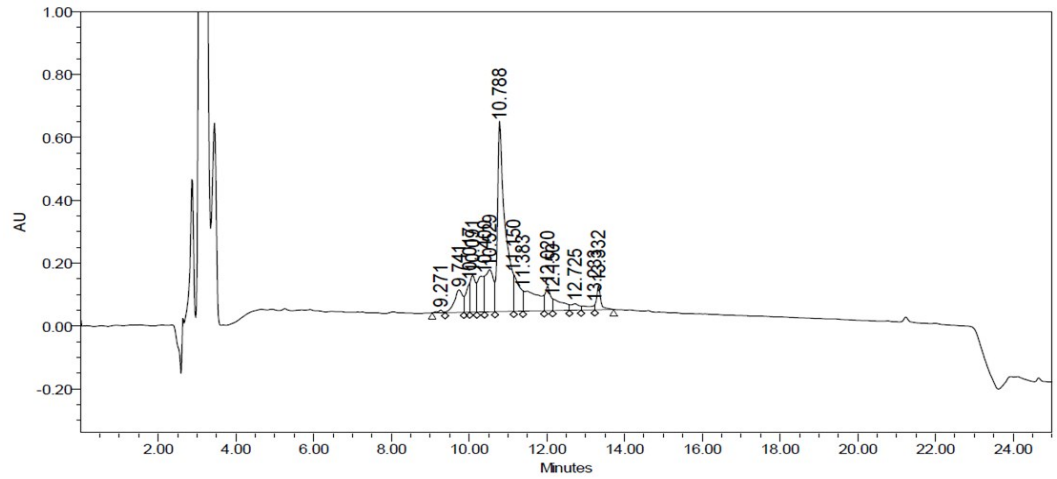


Figure S4. HPLC profile of crude GpTx-1 folded from linear crude peptide organized by stepwise strategy (HPLC Gradients: 10-65% for 20 mins)

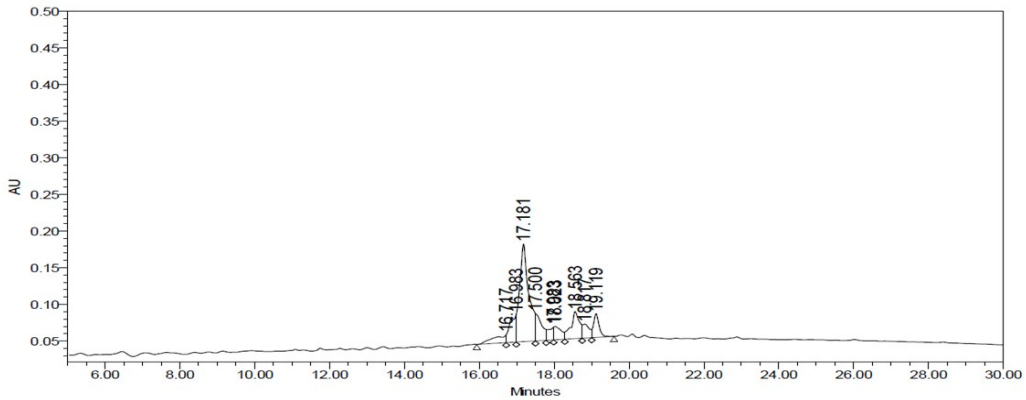


Figure S5. HPLC profile of linear GpTx-1-71 synthesized by stepwise strategy (HPLC Gradient: 10-65% for 30 mins)

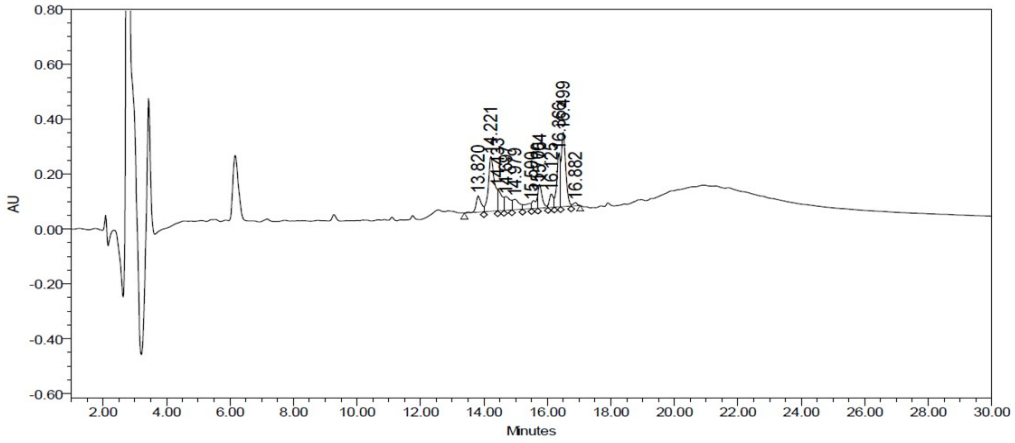


Figure S6. HPLC profile of crude GpTx-1-71 folded from linear crude peptide organized by stepwise strategy (HPLC Gradient: 10-65% for 30 mins)

2.3 Synthesis of H-[20-34]-AM Resin

The substitution of Fmoc-Phe-AM resin was controlled at 0.25-0.55 mmol/g. Linear peptide elongation was performed by solid phase peptide synthesis strategy. Amino acids (2.0 mol equiv), HOBt (3.0mol equiv) and DIC (4.0 mol equiv) were loaded in a container to prepare active ester. To the container, DMF was added and stirred for 5-15 minutes. Pre-activated amino acid mixture was transferred to the appropriate glassware peptide synthesizer equipped with H-Phe-AM resin. Then, the resin mixed with reagents was incubated for 60-120 minutes stirring by N_2 bubbles. After monitoring by Kaiser Test (KT) of the peptide resin indicated for Negative, the liquid phase was drained and the peptide resin was washed with DMF for 3 times. Removal of Fmoc groups was then performed by two sequential incubations in a 20% piperidine in DMF solution for 5 minutes for the first time, and 10～15 minutes for the second times. Subsequently, the reaction mixture was drained and washed with DMF for 5～6 times. The reaction cycle was repeated until the targeting linear peptide resin segment was acquired. A small portion of peptide resin H-[20-34]-AM (～100 mg) is cleaved off in the presence of cleavage cocktails composed of TFA, TIS, H_2O and DODT in the ratio of 90:5:2.5:.2.5 (v/v/v/v). The cleavage reaction was performed at room temperature for 1.5-2.0 hours. After filtration, the desired product was precipitated and dried under vacuum. The purity of H-[20-34]-NH_2 was determined by HPLC analysis. And the molecular weight was determined by ESI-MS (LCMS-8030, shimadzu).

HPLC purity of H-[20-34]-NH_2 (GpTx-1): 89.3% (Figure S7)
(Gradients: 10%-65% mobile phase B for 20 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/CH_3CN; Flow rate: 0.8 mL/min; 215 nm)

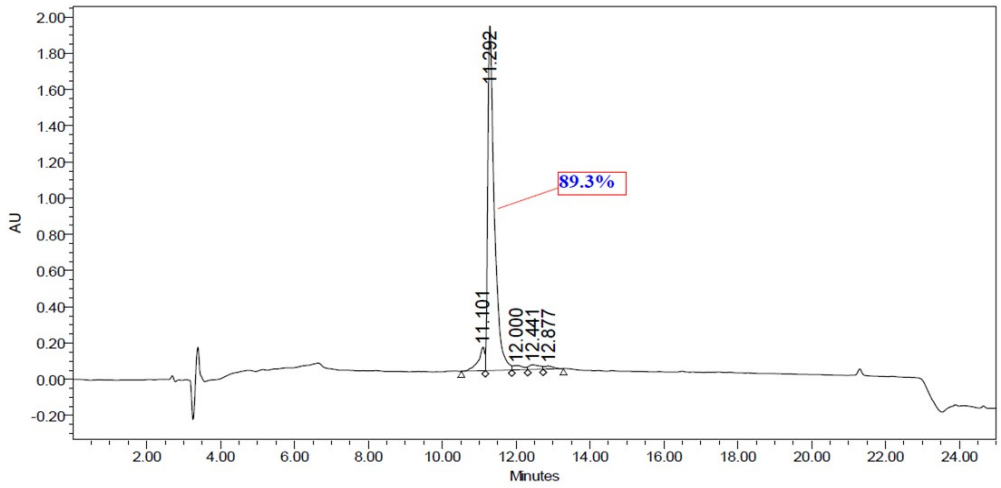


Figure S7. HPLC profile of H-[20-34]-NH_2 (GpTx-1)

ESI-MS (LCMS-8030, shimadzu) of H-[20-34]-NH_2 (GpTx-1, Figure S9)

H-Asn-Leu-Val-Cys-Ser-Arg-Thr-His-Lys-Trp-Cys-Lys-Tyr-Val-Phe-NH_2

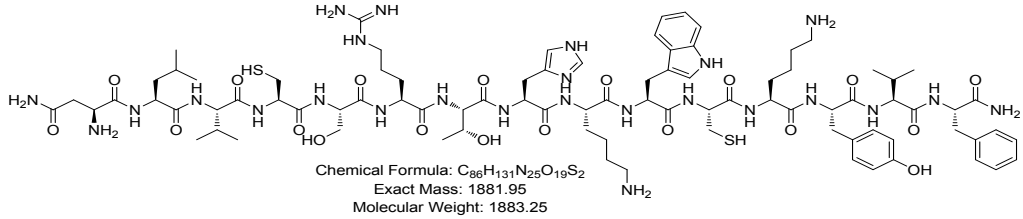


Figure S8 Chemical structure of H-[20-34]-NH_2

Chemical Formula: $C_{86}H_{130}N_{24}O_{20}S_2$; calculated Mw= 1883.25

The peaks with m/z ratios of 942.2, 628.6, 471.9 represent the $[M+2H]^{2+}$, $[M+3H]^{3+}$, $[M+4H]^{4+}$ of the linear segment of GpTx-1 (H-[20-34]-NH_2).

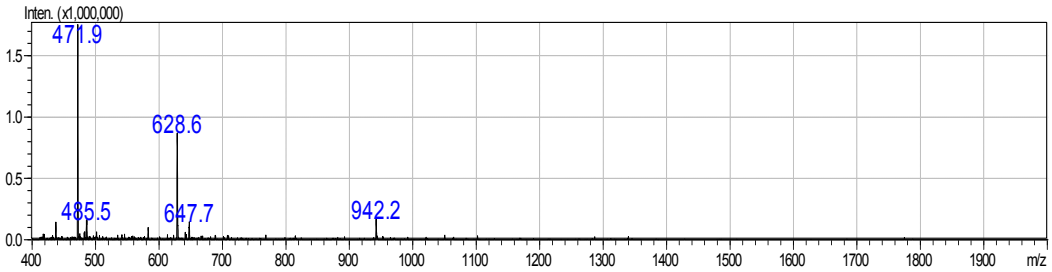


Figure S9. Ms profile of H-[20-34]-NH_2 (GpTx-1)

HPLC purity of H-[20-34]-NH_2: 95.3% (GpTx-1-71, Figure S10)

(Gradients: 10%-65% mobile phase B for 30 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/CH_3CN; Flow rate: 0.8 mL/min; 215 nm)

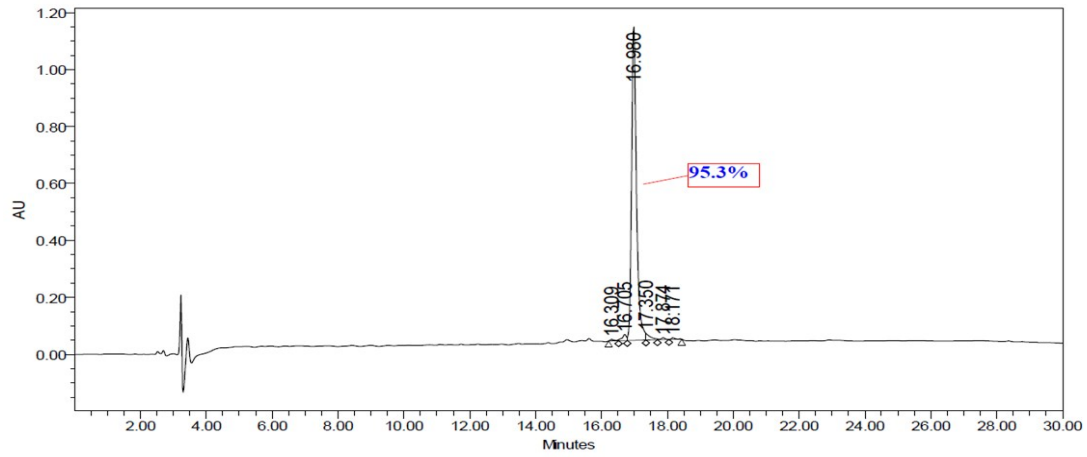
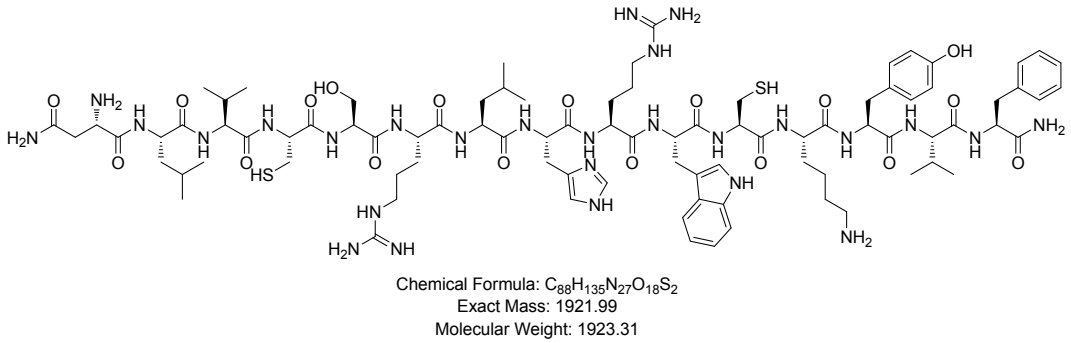


Figure S10. HPLC profile of H-[20-34]-NH_2 (Analogue 71)

ESI-MS (LCMS-8030, shimadzu) of H-[20-34]-NH_2 (GpTx-1-71, Figure S11)



Chemical Formula: $C_{88}H_{135}N_{27}O_{18}S_2$; calculated Mw= 1923.31

The peaks with m/z ratios of 962.3, 642.2, 481.8 represent the $[M+2H]^{2+}$, $[M+3H]^{3+}$, $[M+4H]^{4+}$ of the linear segment of Analogue 71 (H-[20-34]-NH_2).

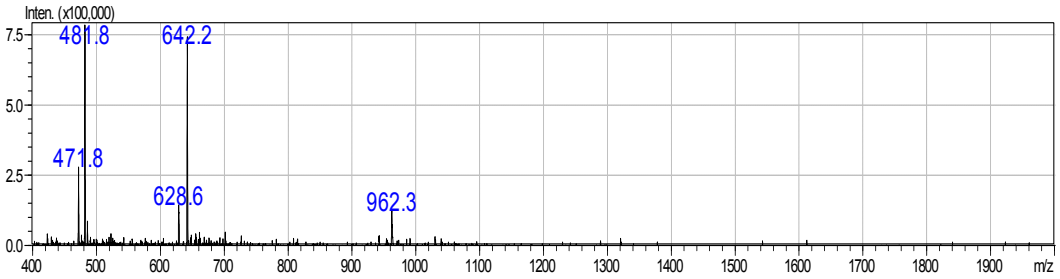


Figure S11. Ms profile of H-[20-34]-NH_2 (GpTx-1-71)

2.4 Synthesis of Fmoc-[12-19]-OH

Fmoc-Asp(tBu)-Asn(Trt)-Asp(OtBu)-Lys(Boc)-Cys(Trt)-Cys(Trt)-Arg(Pbf)-Pro-OH (GpTx-1 & GpTx-1-71)

Fmoc-Pro-CTC resin was synthesized by loading 5.0 g of 2-CTC (1.0 eq. Sub=1.0 mmol/g), Fmoc-Pro-OH (1.5 eq.) and 4.0 eq. of DIEA using DCM/DMF as reaction solvent for 60 minutes. Un-reacted site was quenched by MeOH in the presence of 4.0 eq. of DIEA. After that, linear peptide elongation was performed by stepwise addition using standard solid phase methods. Amino acids (2.0 mol equiv), PyBop (2.0 mol equiv), HOBt (2.0 mol equiv) and DIEA (3.0-3.5 mol equiv) was loaded in a container to prepare active ester. To the container, DMF/DCM mixture solvent was added and stirred for 5～15 minutes. Preactivated amino acid was transferred to the appropriate glassware peptide synthesizer equipped with H-Pro-CTC resin. Then, the resin mixed with reagents was incubated for 60～180 mins stirring by N_2 bubbles. After monitoring by Kaiser Test of the peptide resin indicated for Negative, the liquid phase was drained and the peptide resin was washed with DMF for 3 times. Removal of Fmoc groups was then performed by two sequential incubations in a 20% piperidine in DMF solution for 3～5 minutes for the first time, and 5～15 minutes for the second times. Subsequently, the reaction mixture was drained and washed with DMF for 6 times. The reaction cycle was repeated until the whole linear peptide resin was acquired.

9.6 g of Fmoc-[12-19]-OH is cleaved off from the peptide resin in the presence of 1% TFA/DCM at room temperature for 30～45 min. After filtration off the resin, the resin washed with DCM for 2 times. The combined filtrates were neutralized with 5% $NaHCO_3$ solution. Then the organic phase was washed by saturated NaCl solution for 3 times. The organic phase was collected and concentrated under vacuum to give a white solid (4.7 g) with a 97.9% HPLC purity in 39.7% overall yield. The product was employed as the starting materials for the next coupling step directly.

HPLC purity of Fmoc-[12-19]-OH: 97.9% (Figure S12, Segment of GpTx-1 and GpTx-1-71) (Gradients: 70%-95% mobile phase B for 20 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/CH_3CN; Flow rate: 0.8 mL/min; 215 nm)

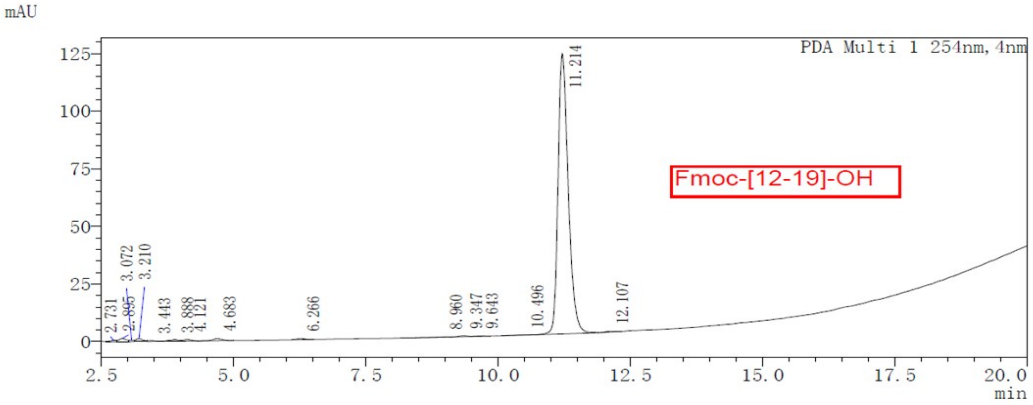


Figure S12. HPLC profile of Fmoc-[12-19]-OH (Segment of GpTx-1 and GpTx-1-71)

MS (ABI Q-star Elite) of Fmoc-[12-19]-OH (Figure S14, GpTx-1 and GpTx-1-71)

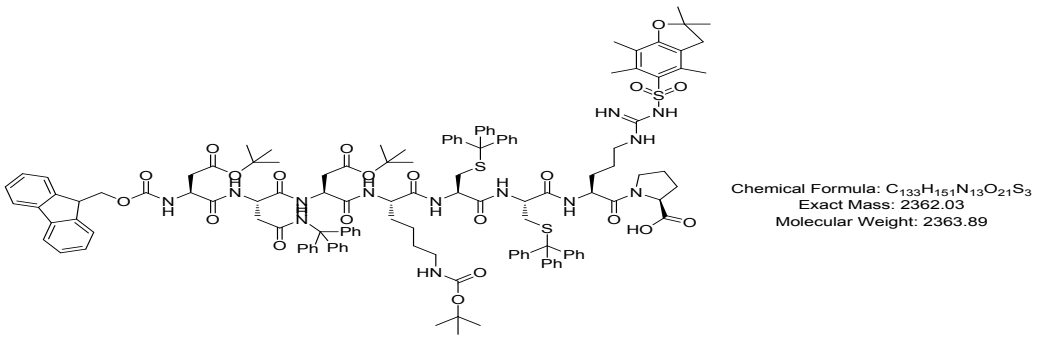


Figure S13 Chemical structure of Fmoc-[12-19]-OH

The peaks with m/z ratios of 2363.0403, 2385.0381 represent the $[M+H]^{1+}$, $[M+Na]^{1+}$of the linear segment (Fmoc-[12-19]-OH).

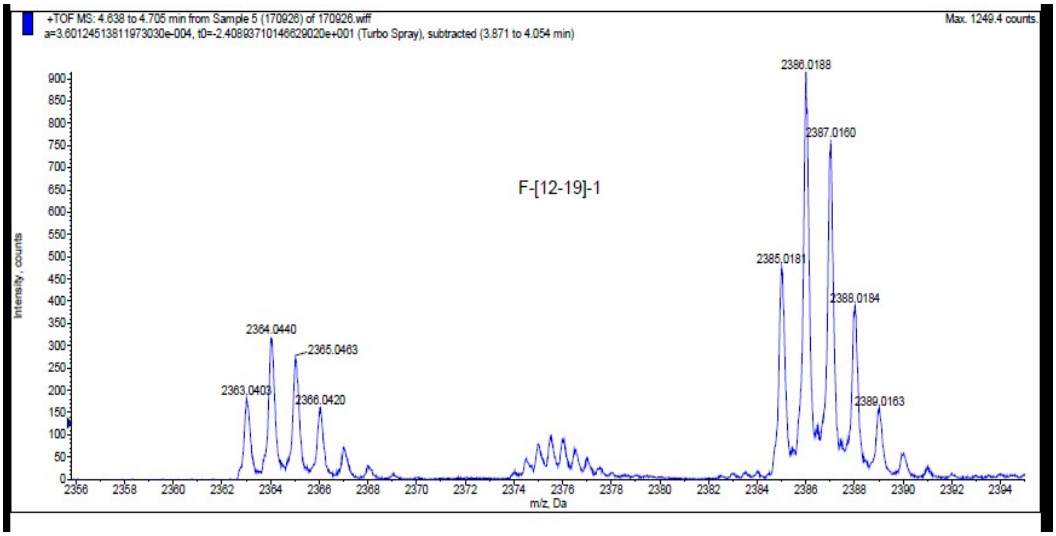


Figure S14. Ms profile of Fmoc-[12-19]-OH (Segment of GpTx-1 and GpTx-1-71)

2.5 Synthesis of Boc-[1-11]-OH or Fmoc-[1-11]-OH

Fmoc-Pro-CTC resin was synthesized by loading 2-Cl-CTC (1.0 eq. Sub=1.0 mmol/g), Fmoc-Pro-OH (1.5 eq.) and 4.0 eq. of DIEA using DCM/DMF as reaction solvent. Un-reacted site was quenched by MeOH in the presence of 4.0 eq. of DIEA.

Linear peptide elongation was performed by stepwise addition using solid phase peptide synthesis methods. H-Pro-CTC (2.3 g, 1.4 mmol), amino acid (2.0 mol equiv), HOBt (2.0 mol equiv), PyBop (2.0 eq.) and DIEA (3.0-3.5 mol equiv) was loaded in a container to prepare active ester. To the container, DMF/DCM mixture solvent was added and stirred for 5～15 minutes. Preactivated amino acid was transferred to the appropriate glassware peptide synthesizer equipped with H-Pro-CTC resin. Then, the resin mixed with reagents was incubated for 1-3 hours stirring by N_2 bubbles. After monitoring by Kaiser Test of the peptide resin indicated for Negative, the liquid phase was drained and the peptide resin was washed with DMF for 3 times. Removal of Fmoc groups was then performed by two sequential incubations in a 20% piperidine in DMF solution for 3～5 minutes for the first time, and 5～15 minutes for the second times. Subsequently, the reaction mixture was drained and washed with DMF for 6

times. The reaction cycle was repeated until the whole linear peptide resin was acquired. After drying, 3.9 g of peptide resin was obtained.

3.9 g of Boc-[1-11]-CTC (or Fmoc-[1-11]-CTC resin, GpTx-1) is cleaved off in the presence of 1% TFA/DCM for 30～45 min. After filtration off the resin, the resin washed with DCM for 2 times. The combined filtrates were neutralized with 5% $NaHCO_3$ solution. Then the organic phase was washed by saturated NaCl solution for 3 times. It was collected and concentrated under vacuum to give 1.9 g a white solid with 90.1% HPLC purity (Figure S15) in 56.6% yield. The product was no necessary for further purification and employed as the starting materials for the next coupling step directly.

HPLC purity of Boc-[1-11]-OH (GpTx-1, Figure S15)
(Gradients: 70%-95% mobile phase B for 20 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/CH_3CN; Flow rate, 0.8 mL/min; 215 nm)

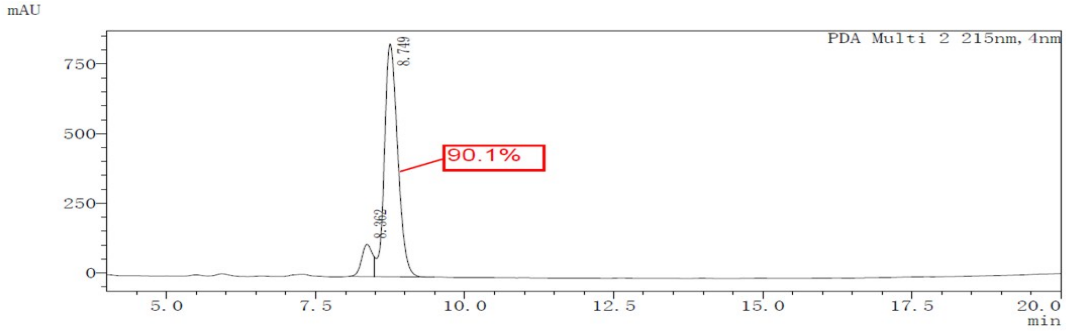
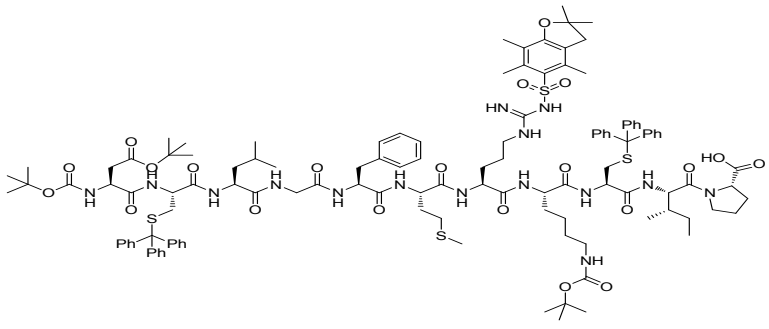


Figure S15. HPLC profile of Boc-[1-11]-OH (GpTx-1)

ESI-MS (ABI Q-star Elite) of Ms of Boc-[1-11]-OH (GpTx-1, Figure S16)
Boc-Asp(O′Bu)-Cys(Trt)-Leu-Gly-Phe-Met-Arg(Pbf)-Lys(Boc)-Cys(Trt)-Ile-Pro-OH



Chemical Formula: $C_{120}H_{159}N_{15}O_{21}S_4$
Exact Mass: 2274.07
Molecular Weight: 2275.89

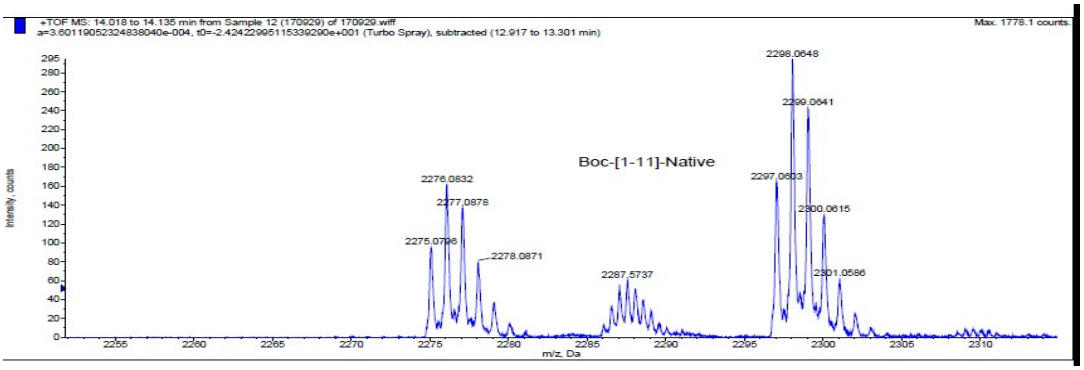


Figure S16. Ms profile of Boc-[1-11]-OH (GpTx-1)

The peaks with m/z ratios of 2275.0796, 2297.0603 represent the $[M+H]^{1+}$, $[M+Na]^{1+}$of the linear segment (Boc-[1-11]-OH).

HPLC purity of Boc-[1-11]-OH (GpTx-1-71, Figure S17)
(Gradients: 70%-95% mobile phase B for 20 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/CH_3CN; 0.8 mL/min; 215 nm)

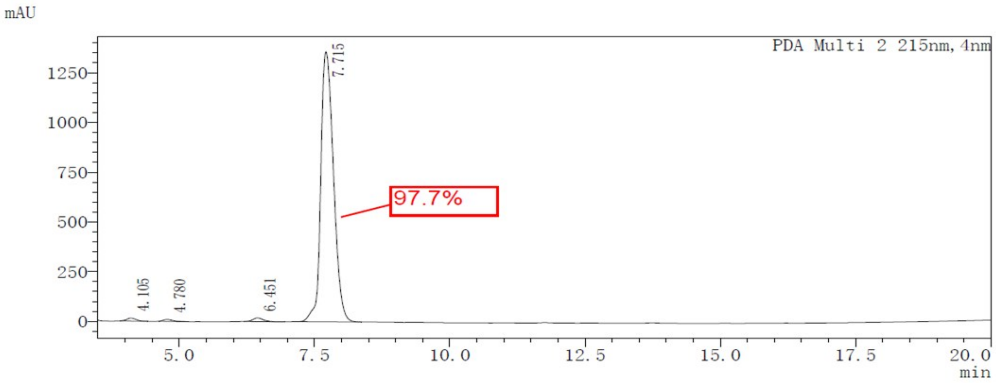
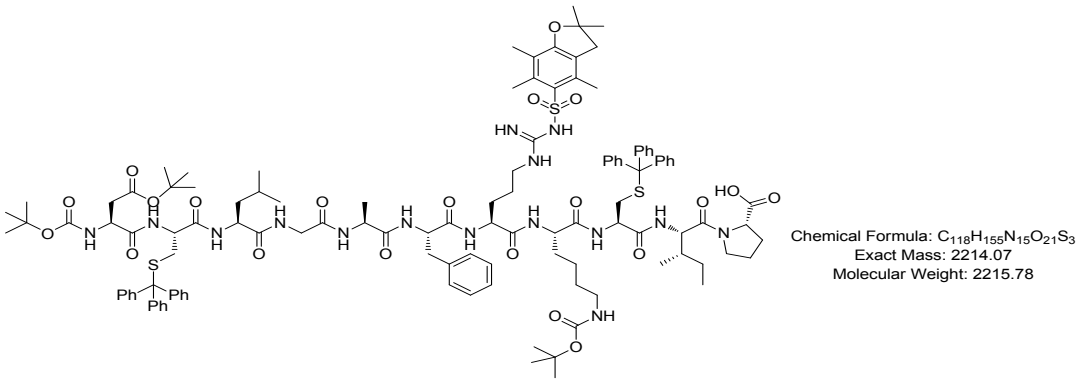


Figure S17. HPLC profile of Boc-[1-11]-OH (GpTx-1-71)

Ms (ABI Q-star Elite) of Boc-[1-11]-OH (GpTx-1-71, Figure S18)
Boc-Asp(O^{t}Bu)-Cys(Trt)-Leu-Gly-Ala-Phe-Arg(Pbf)-Lys(Boc)-Cys(Trt)-Ile-Pro-OH



The peaks with m/z ratios of 2215.0759, 2237.0557 represent the $[M+H]^{1+}$, $[M+Na]^{1+}$of the linear segment (Boc-[1-11]-OH).

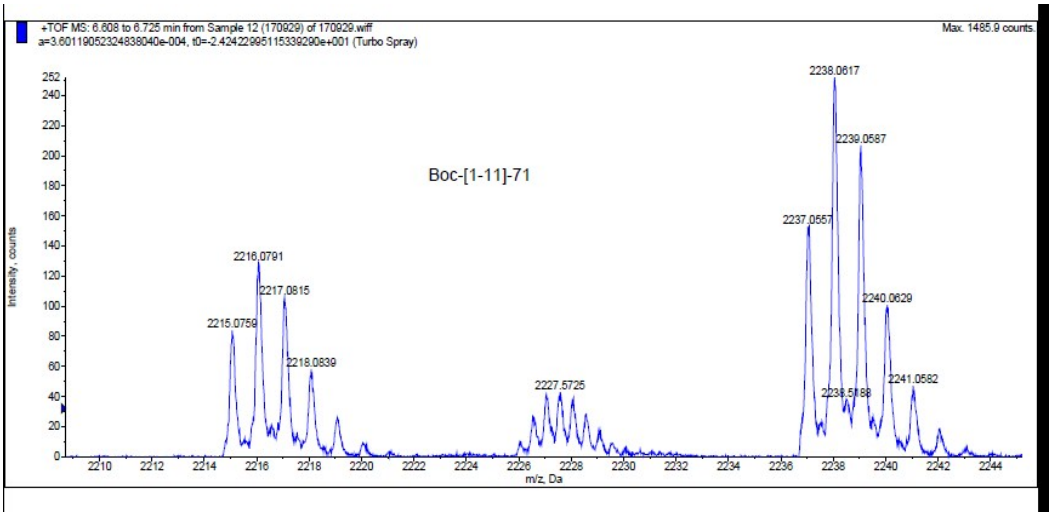


Figure S18. Ms profile of Boc-[1-11]-OH (GpTx-1-71)

HPLC purity of Fmoc-[1-11]-OH (GpTx-1, Figure S19)

(Gradients: 70%-95% mobile phase B for 20 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/CH_3CN; 0.8 mL/min)

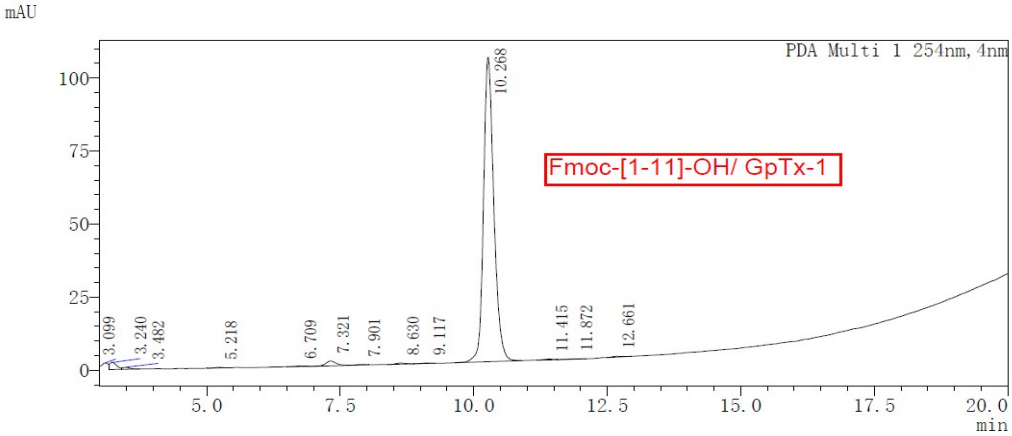
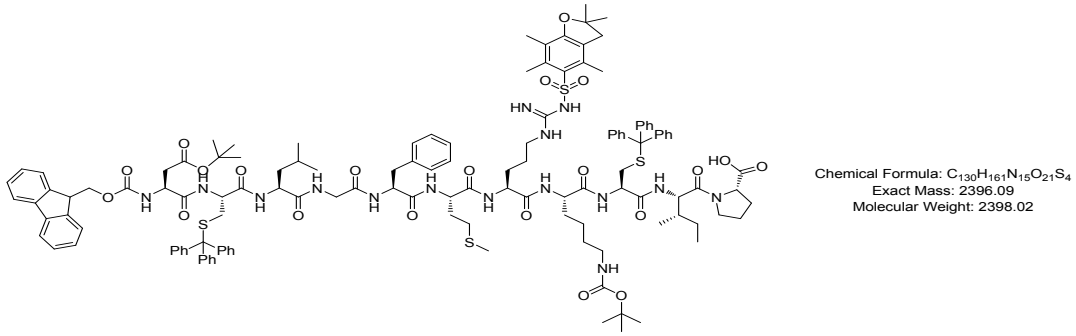


Figure S19. HPLC profile of Fmoc-[1-11]-OH (GpTx-1)

Ms (ABI Q-star Elite) of Fmoc-[1-11]-OH (GpTx-1, Figure S20)



The peaks with m/z ratios of 2397.0906, 2419.0757 represent the $[M+H]^{1+}$, $[M+Na]^{1+}$of the linear segment (Fmoc-[1-11]-OH).

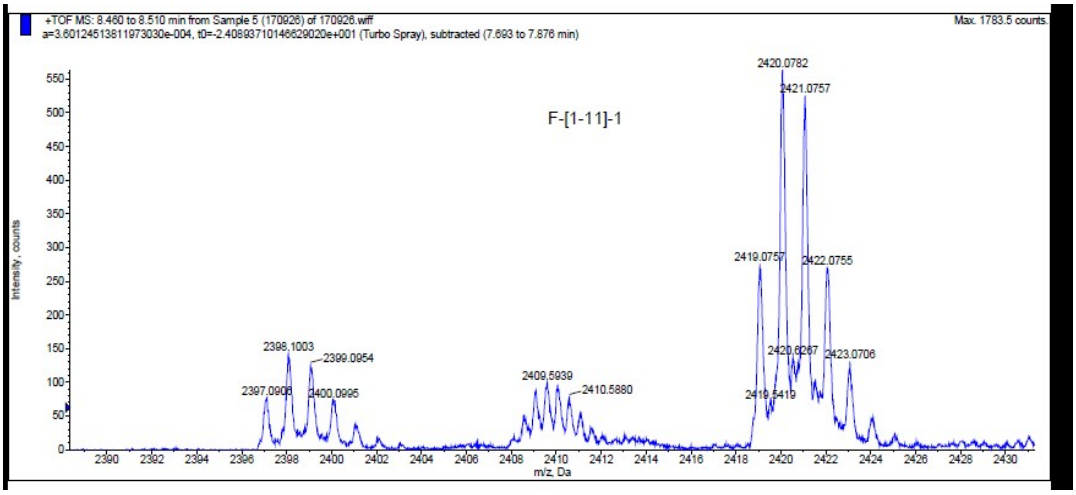


Figure S20. Ms profile of Fmoc-[1-11]-OH (GpTx-1)

HPLC purity of Fmoc-[1-11]-OH (GpTx-1-71, Figure S21)
(Gradients: 70%-95% mobile phase B for 20 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/CH_3CN; 0.8 mL/min; 215 nm)

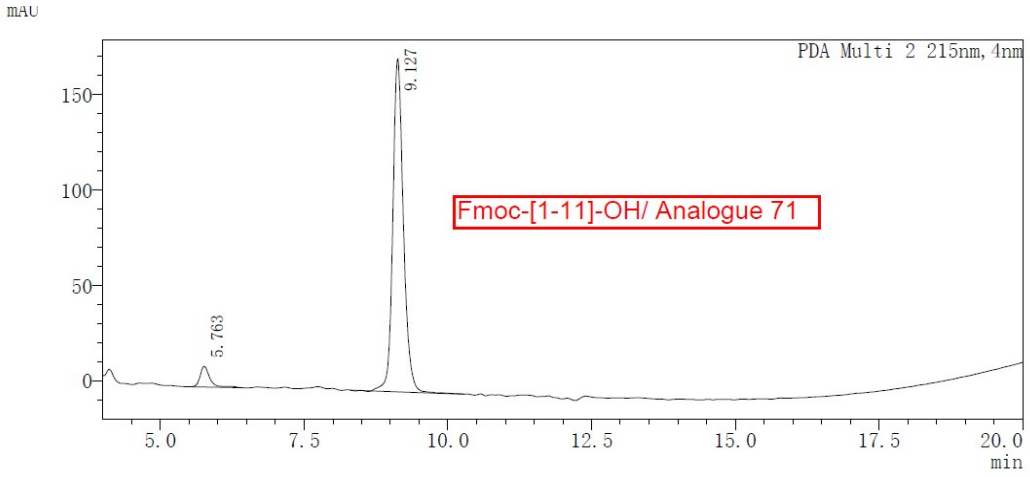
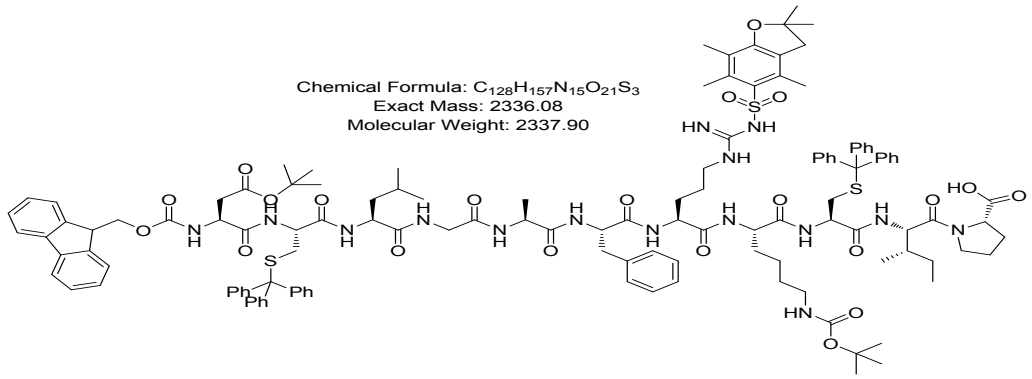


Figure S21. HPLC profile of Fmoc-[1-11]-OH (GpTx-1-71)

Ms (ABI Q-star Elite) of Fmoc-[1-11]-OH (GpTx-1-71, Figure S22)



The peaks with m/z ratios of 2359.0731 represent the $[M+Na]^{1+}$ of the linear segment (Fmoc-[12-19]-OH).

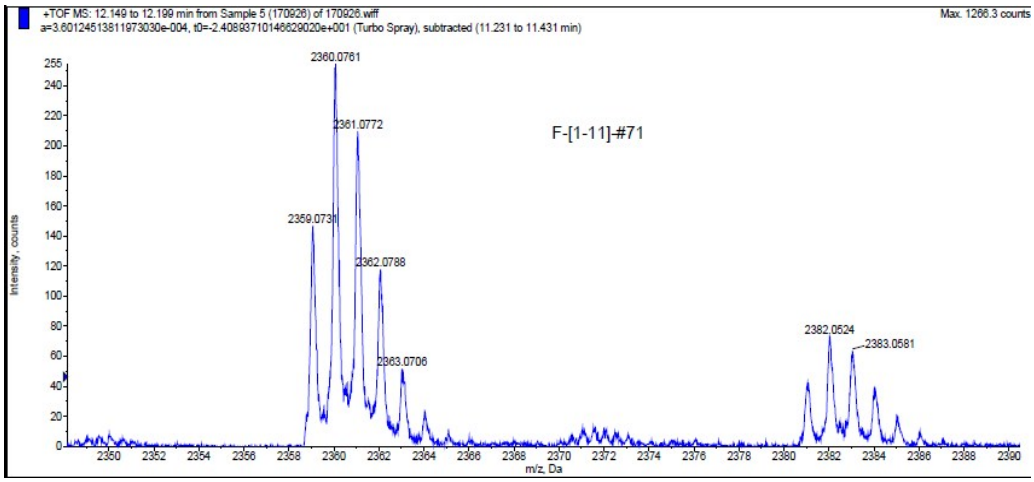


Figure S22. Ms profile of Fmoc-[1-11]-OH (GpTx-1-71)

2.6 Synthesis of H-[11-34]-AM Resin

3.3 g of H-[20-34]-AM resin (1.0 eq.; 0.6 mmol), HOBt (4.5 eq.) and Fmoc-[12-19]-OH (1.5 eq.) was loaded to a 100 mL flask. After addition of DCM/DMF, DIC (6.0 eq.) was added to the flask. The reaction was stirring at room temperature for 3-6 hrs. After KT indicated the peptide resin colorless, it was filtered and washed with DMF for 3 times. Then removal of Fmoc group was treated with 20% PIP for 5+10 minutes. A small portion of peptide resin (～100 mg) is cleaved off and the acquired product was used for HPLC detection and Ms identification.

HPLC purity of H-[12-34]-OH (GpTx-1): 76.8% (Figure S23)
(Gradients: 10%-65% mobile phase B for 30 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/CH_3CN; 0.8 mL/min; 215 nm)

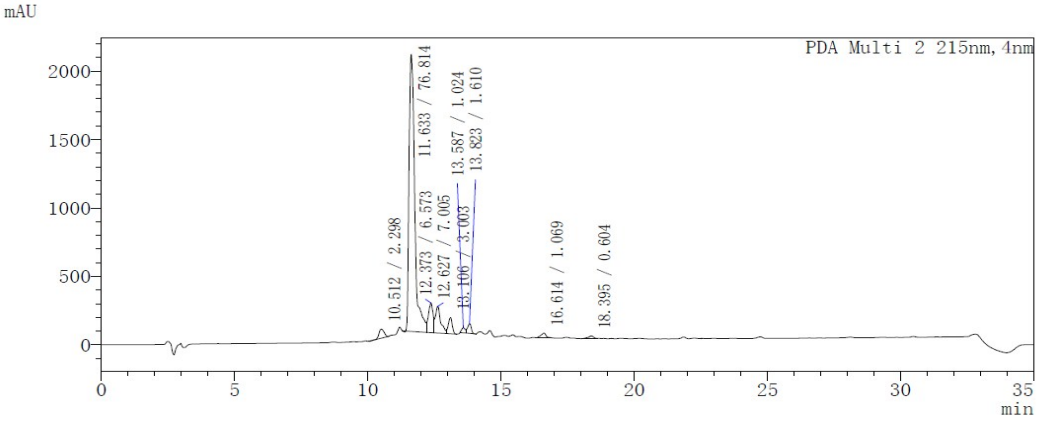
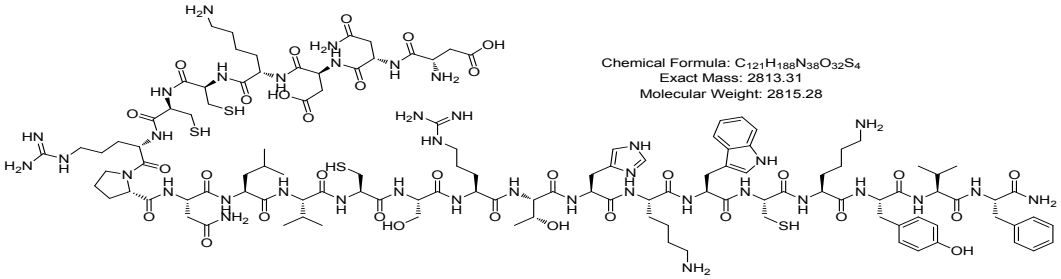


Figure S23. HPLC profile of H-[12-34]-NH_2 (GpTx-1)

ESI-MS (LCMS-8030, shimadzu) of H-[12-34]-NH_2 (GpTx-1, Figure S24)

H-Asp-Asn-Asp-Lys-Cys-Cys-Arg-Pro-Asn-Leu-Val-Cys-Ser-Arg-Thr-His-Lys-Trp-Cys-Lys-Tyr-Val-Phe-NH_2



Chemical Formula: $C_{131}H_{188}N_{38}O_{32}S_4$; calculated Mw=2815.28

The peaks with m/z ratios of 1408.30, 939.45, 704.80, 564.10 represent the $[M+2H]^{2+}$, $[M+3H]^{3+}$, $[M+4H]^{4+}$, $[M+5H]^{5+}$of the linear segment of GpTx-1 (H-[20-34]-NH_2).

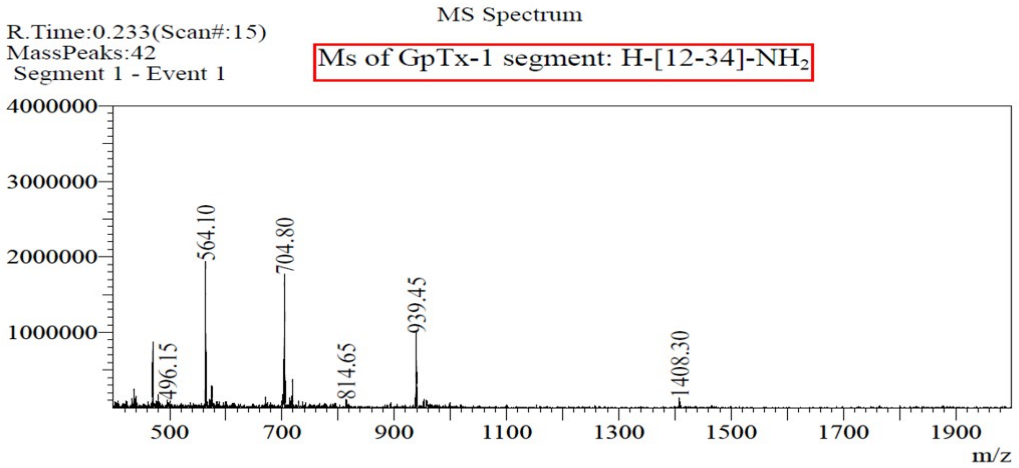
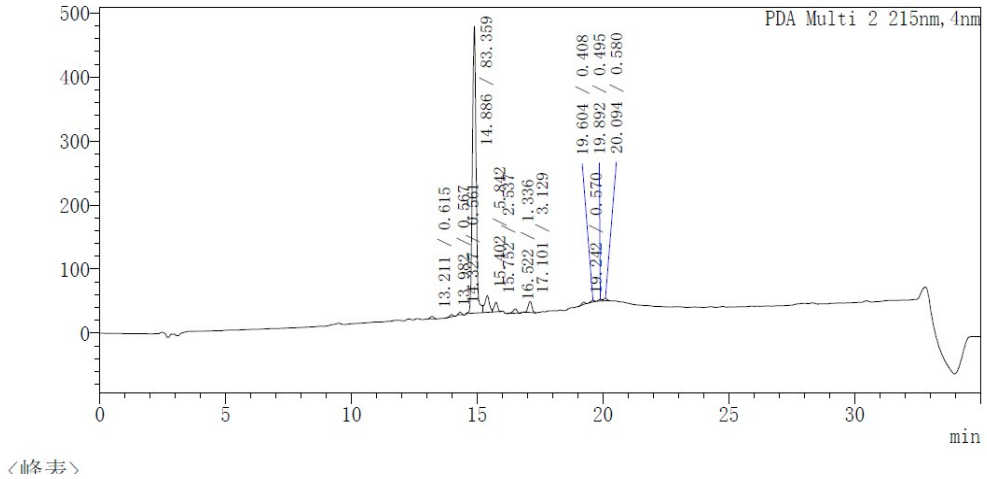


Figure S24. Ms profile of H-[12-34]-NH_2 (GpTx-1)

HPLC purity of H-[12-34]-NH_2 (GpTx-1-71): 83.4% (Figure S25)

(10%-65% for 30 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/ CH_3CN)

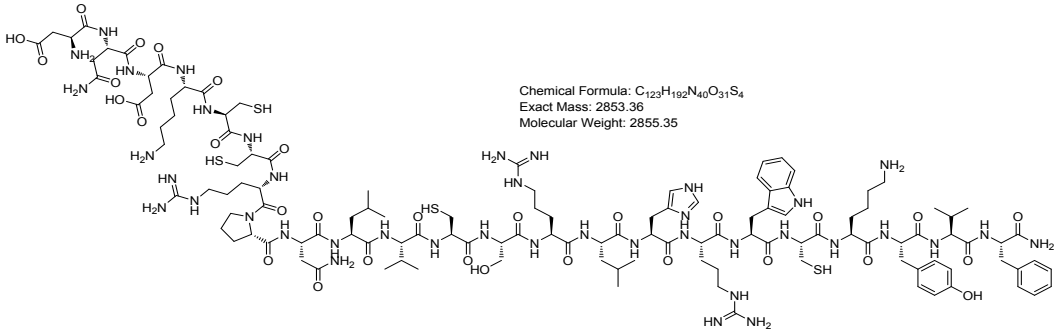


〈峰表〉

Figure S25. HPLC profile of H-[12-34]-NH_2 (GpTx-1-71)

ESI-MS (LCMS-8030, shimadzu) of H-[12-34]-NH_2 (GpTx-1-71, Figure S26)

H-Asp-Asn-Asp-Lys-Cys-Cys-Arg-Pro-Asn-Leu-Val-Cys-Ser-Arg-Leu-His-Arg-Trp-Cys-Lys-Tyr-Val-Phe-NH_2



Chemcial Formula: $C_{123}H_{192}N_{40}O_{31}S_4$; Mw: 2855.35

1428.65=$[TM+2]^{2+}$; 952.65=$[TM+3]^{3+}$; 714.85=$[TM+4]^{4+}$; 572.10=$[TM+5]^{5+}$.

The peaks with m/z ratios of 1428.65, 952.65, 714.85, 572.10 represent the $[M+2H]^{2+}$, $[M+3H]^{3+}$, $[M+4H]^{4+}$, $[M+5H]^{5+}$of the linear segment of GpTx-1 (H-[20-34]-NH_2).

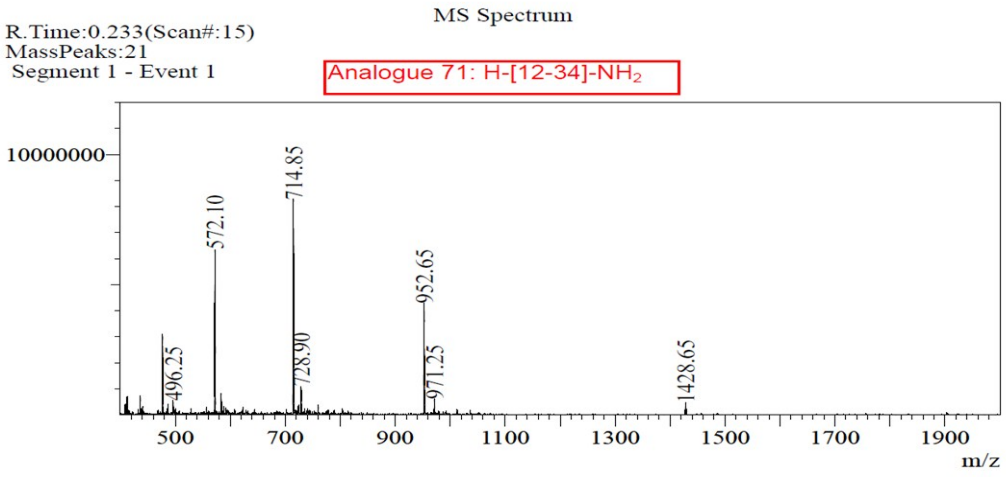


Figure S26. Ms profile of H-[12-34]-NH_2 (GpTx-1-71)

2.7 Synthesis of Boc-[1-34]-AM Resin (or Fmoc-[1-34]-AM Resin)

3.0 g of H-[12-34]-AM resin (1.0 eq., 0.45 mmol), HOBt (4.5 eq.) and Boc-[1-11]-OH/or Fmoc-[1-11]-OH (1.5 eq.) was loaded to a 100 mL flask. After addition of DCM/DMF, DIC (6.0 eq.) was added to the flask. The reaction was stirring at room temperature for 3-6 hrs. After KT indicated the peptide resin colorless, the peptide resin was filtered and washed with DMF for 3 times. Then removal of Fmoc group was treated with 20% PIP for 5+ 10 minutes (Only for Fmoc-protected segment). The linear peptide resin was dried under vacuum at room temperature.

2.8 H-[1-34]-NH_2 Cleavage off form the peptide resin

To the peptide resin (1.0 g GpTx-1) was added TIS/DODT/H_2O/TFA (5.0: 2.5: 2.5: 9.0 = V/V/V/V). The cleavage reaction was performed for 1.5-2.0 h at room temperature. Then the cleavage mixture was filtered and the collected filtrate was added to a low polarity solvent MTBE to precipitate the product. After white precipitate formed, the mixture was agitated with the pipet to ensure complete mixing and precipitation. The suspension was centrifuged and the organic phase was discarded. Then, MTBE was added to the tube contained to wash the product for 3 times. The crude linear product was dried under vacuum to give 420 mg a slight yellow solid with 71.8% HPLC purity (Figure S27). GpTx-1-71 (1.0 g linear peptide resin) was cleaved off using the sampe procedure with GpTx-1. At last, 480 mg crude linear peptide Analogue 71 was obtained with 72.4% HPLC purity (Figure S29).

HPLC purity of H-[1-34]-NH_2 (GpTx-1): 71.8% (Figure S27)
(10%-65% for 30 min; Mobile phase A, 10%-65% for 30 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/CH_3CN)

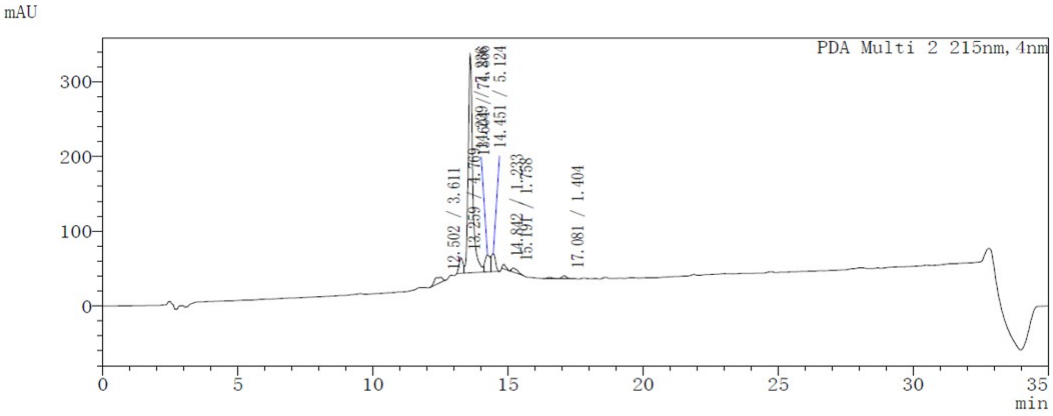
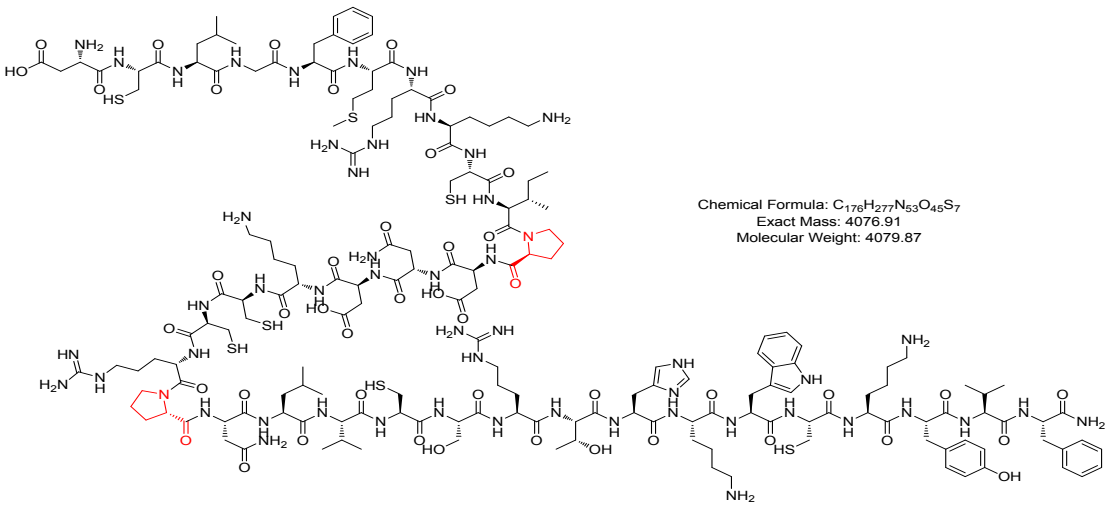


Figure S27. HPLC profile of H-[1-34]-NH_2 (GpTx-1)

ESI-MS Ms of linear GpTx-1: H-[1-34]-NH_2 (Figure S28)



Chemical Formula: $C_{176}H_{277}N_{53}O_{45}S_7$; calculated Mw=4079.87

1360.60=$[TM+3]^{3+}$; 1020.90=$[TM+4]^{4+}$; 816.95=$[TM+5]^{5+}$; 681.05=$[TM+6]^{6+}$; 583.90=$[TM+7]^{7+}$; 511.05= $[TM+8]^{8+}$;

The peaks with m/z ratios of 1360.60, 1020.90, 816.95, 681.05, 583.90 represent the $[M+3H]^{3+}$, $[M+4H]^{4+}$, $[M+5H]^{5+}$, $[M+6H]^{6+}$, $[M+7H]^{7+}$of the linear targeting product GpTx-1.

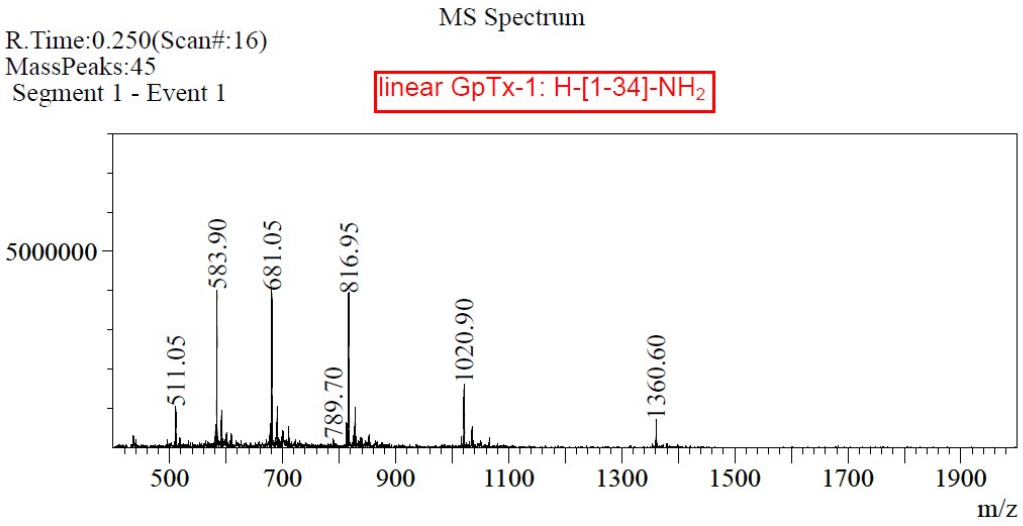


Figure S28. MS profile of linear H-[1-34]-NH_2 (GpTx-1)

ESI-MS (LCMS-8030, shimadzu) analysis of the linear crude product was showed in Figure 24. The peaks with m/z ratios of 1354.50, 1015.70, 813.05, 677.55, 581.05 represent the $[M+3H]^{3+}$, $[M+4H]^{4+}$, $[M+5H]^{5+}$, $[M+6H]^{6+}$, $[M+7H]^{7+}$of the linear targeting product analogue 71. The MS data indicated the major products meet with the expected molecular masses as shown in the spectra below.

HPLC purity of H-[1-34]-NH_2 (GpTx-1-71, Figure S29)

(10%-65% for 30 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/CH_3CN)

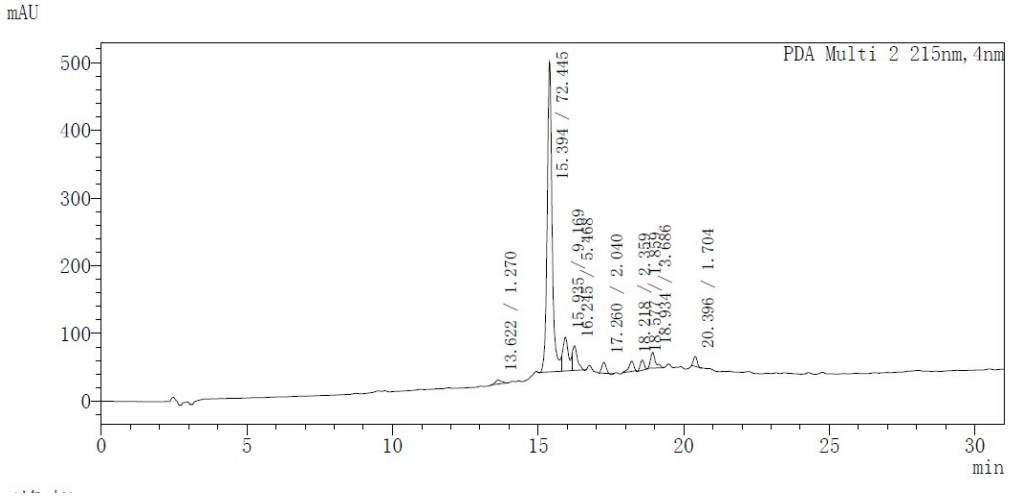
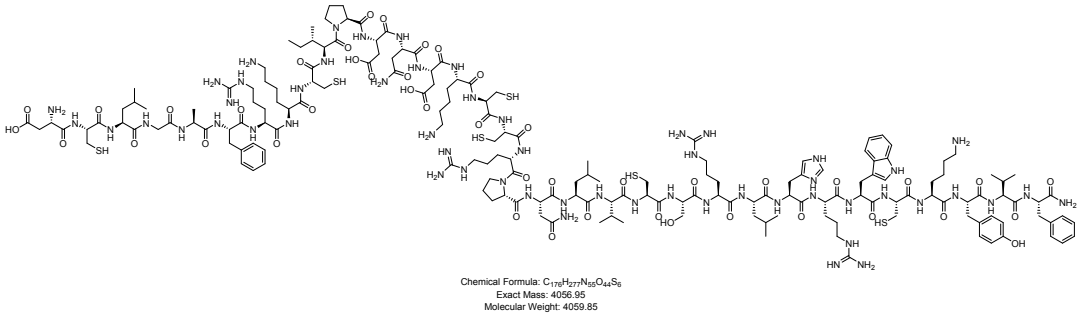


Figure S29. HPLC profile of H-[1-34]-NH_2 (GpTx-1-71)

Ms of GpTx-1-71: H-[1-34]-NH2 (Figure S30)



$C_{176}H_{276}N_{54}O_{45}S_6$; calculated Mw=4059.85

1354.50=$[TM+3]^{3+}$; 1015.70=$[TM+4]^{4+}$; 813.05=$[TM+5]^{5+}$; 677.55=$[TM+6]^{6+}$;; 581.05= $[TM+7]^{7+}$; 508.55= $[TM+8]^{8+}$;

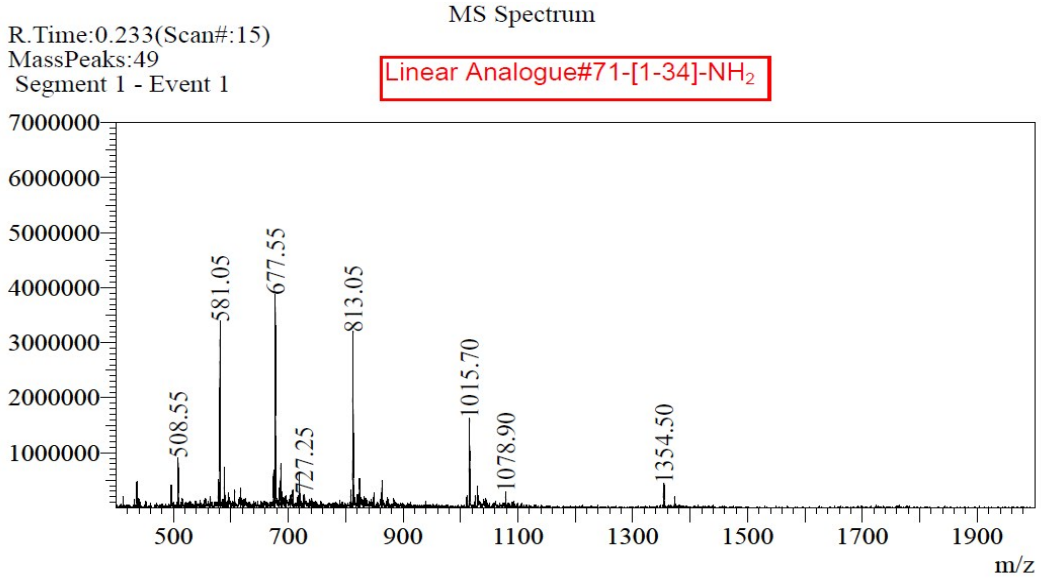


Figure S30. MS profile of linear H-[1-34]-NH_2 (GpTx-1-71)

2.9 Folding of GpTx-1 (Figure S31) and GpTx-1-71 (Figure S32)

In a separate 400 mL of folded buffer by combing 330 mL H_2O, 30 mL ACN, 200 mg oxidized glutathione, 100 mg reduced glutathione, and 40 mL of 1 M Tris-HCl pH 7.5-8.0 was mixed until the solid completely dissolved. Then, the crude petide (420 mg, GpTx-1) was added. The pH of the folding solutions was controlled to be about 7.5-8.0. The reaction was allowed to stand overnight and the progress of the reaction was monitored by HPLC. After the linear peptide consumed completely detected form HPLC, 10 mL of glacial acetic acid was added to quench the reaction.

(Gradients, 10%-65% Mobile phase B for 30 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/CH_3CN; 0.8 mL/min; 215 nm)

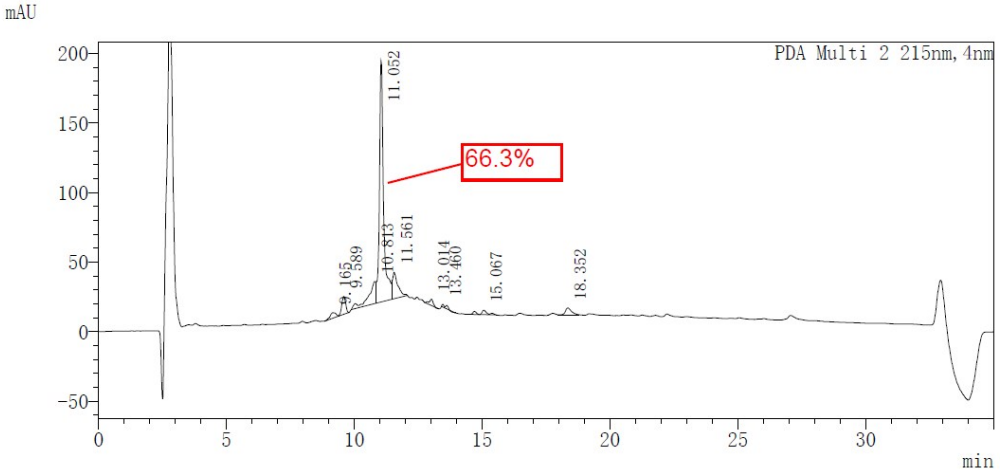


Figure S31. HPLC profile of crude GpTx-1 (reaction mixture)

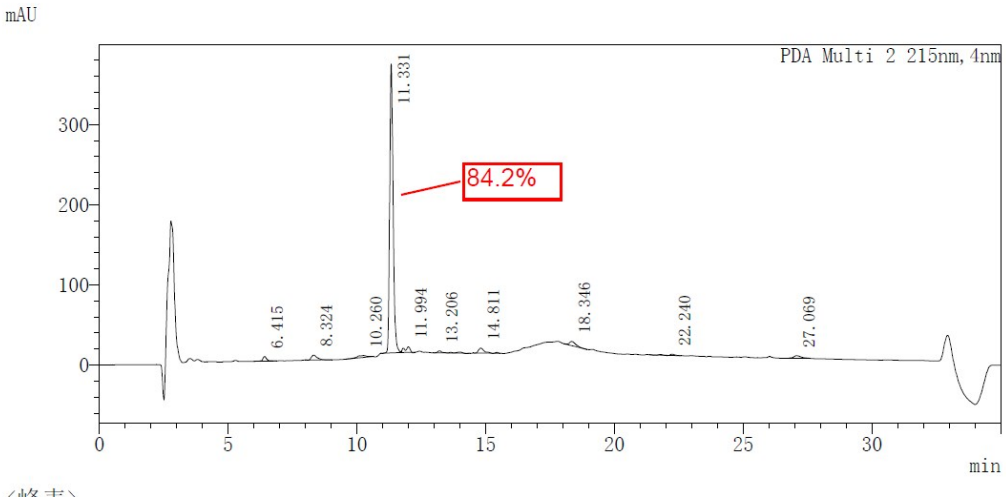


Figure S32. HPLC profile of crude GpTx-1-71 (reaction mixture)

2.10 Purification of GpTx-1 and GpTx-1-71

The reaction mixture was filtered using 0.22 µm membrane filter. The filtrate was loaded on RP-HPLC for purification using C18, 21.2×250 mm, 10 µm, 170 Å column eluted with a 10 to 60% B over 60 min gradient (A, H_2O; B, ACN; mobile phase 0.1 % TFA in each and monitoring absorbance at 220 nm, flow rate: 15 mL/min). Peptide fractions with >95% HPLC purity with correct m/z ratio were collected and lyophilzed, then stored at −20 °C. 32 mg of GpTx-1 was obtained with 98.5% HPLC purity in 7.5% yield based on linear peptide. The highly purified GpTx-1-71 (>98% HPLC purity) was aslo obatained in 3.5% yield. The folded products of GpTx-1 and analogue 71 have the monoisotopic molecular weights of 4050.9145 Da (Figure S35) and 4070.8668 Da (Figure S36) respectively which meet the calculated values accordingly.

HPLC profile of purified GpTx-1 (Figure S33)
(Gradients, 10%-65% Mobile phase B for 30 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/CH_3CN; 0.8 mL/min; 215 nm)

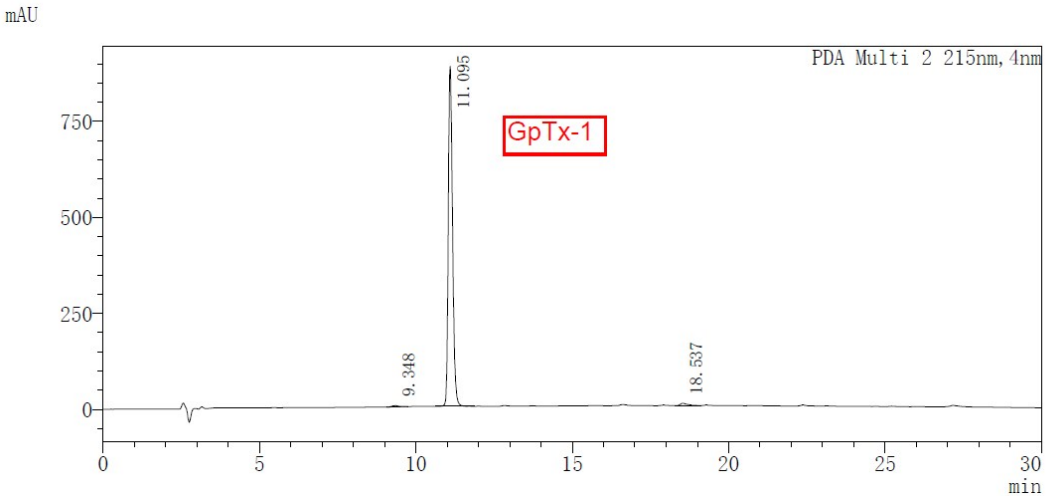


Figure S33. HPLC profile of purified GpTx-1

HPLC profile of purified GpTx-1-71 (Figure S34)
(Gradients, 10%-65% Mobile phase B for 30 min; Mobile phase A: 0.1% TFA/H_2O, Mobile phase B: 0.1% TFA/CH_3CN; 0.8 mL/min; 215 nm)

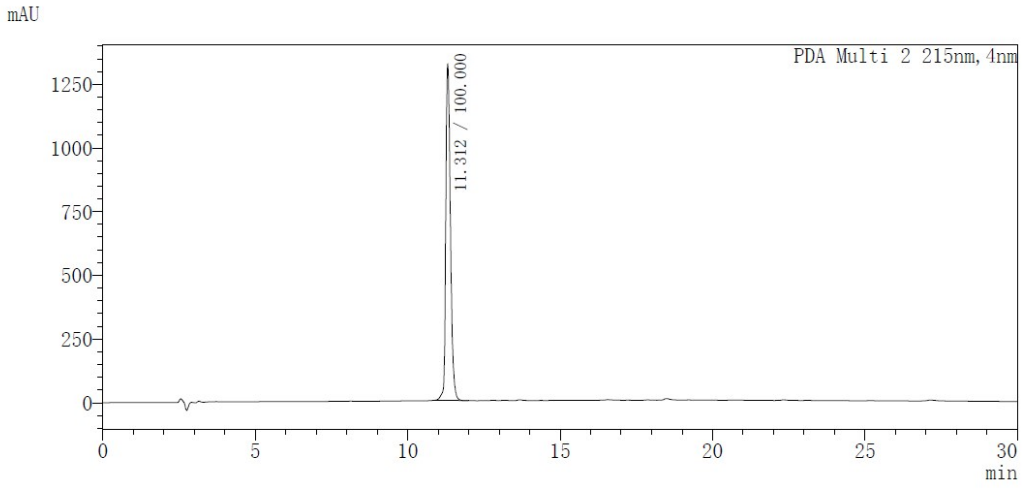


Figure S34. HPLC profile of purified GpTx-1-71

2.11 High resolution Ms of GpTx-1 and GpTx-1-71

ESI-MS of GpTx-1 (Figure S35)

Chemical Formula: $C_{176}H_{271}N_{53}O_{45}S_7$ Exact Mass: 4070.86

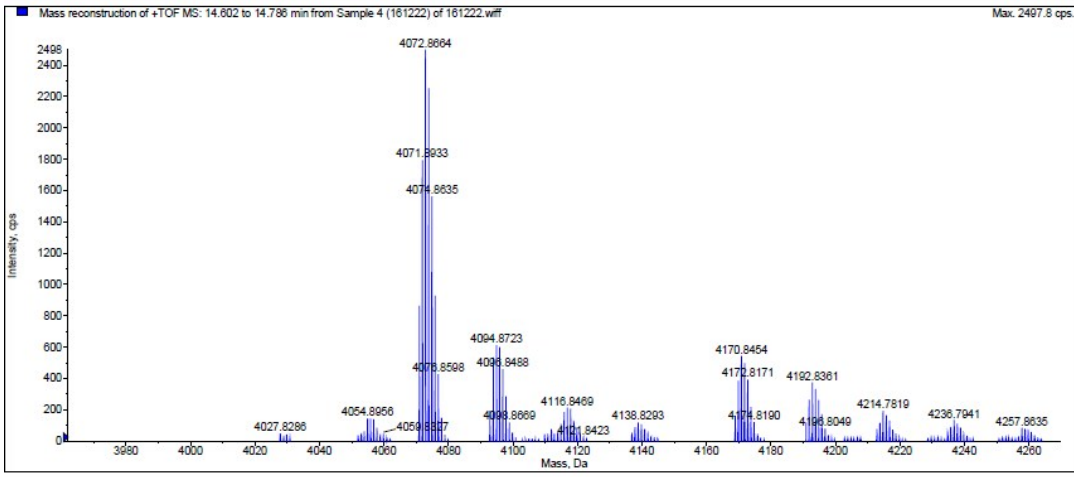


Figure S35. MS profile of GpTx-1

ESI-MS of GpTx-1-71 (Figure S36)

Chemical Formula: $C_{176}H_{271}N_{55}O_{44}S_6$ Exact Mass: 4050.90

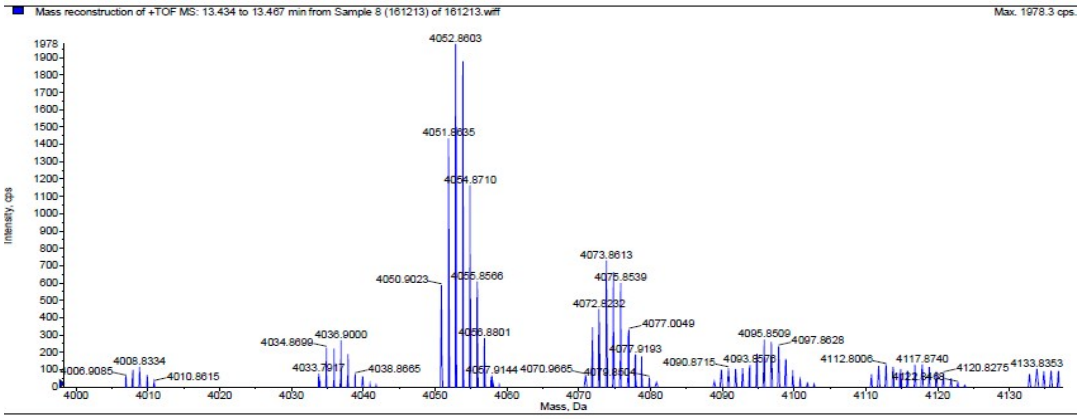


Figure S36. MS profile of of GpTx-1-71

2.12 Manual patch clamp electrophysiology of GpTx-1(#1) and GpTx-1-71 (#2)

The $Na_v1.7$ expressing HEK293 cells were tested. The purified synthetic toxins GpTx-1 and GpTx-1-71 inhibited the Nav1.7 channel currents in a concentration-dependent manner (0.1 nM, 10 nM, 30 nM, 50 nM, 300 nM), with an IC50 of 31 nM and 14 nm which were similar to the previously reported values of 90 nM and 1.6 nM accordingly.

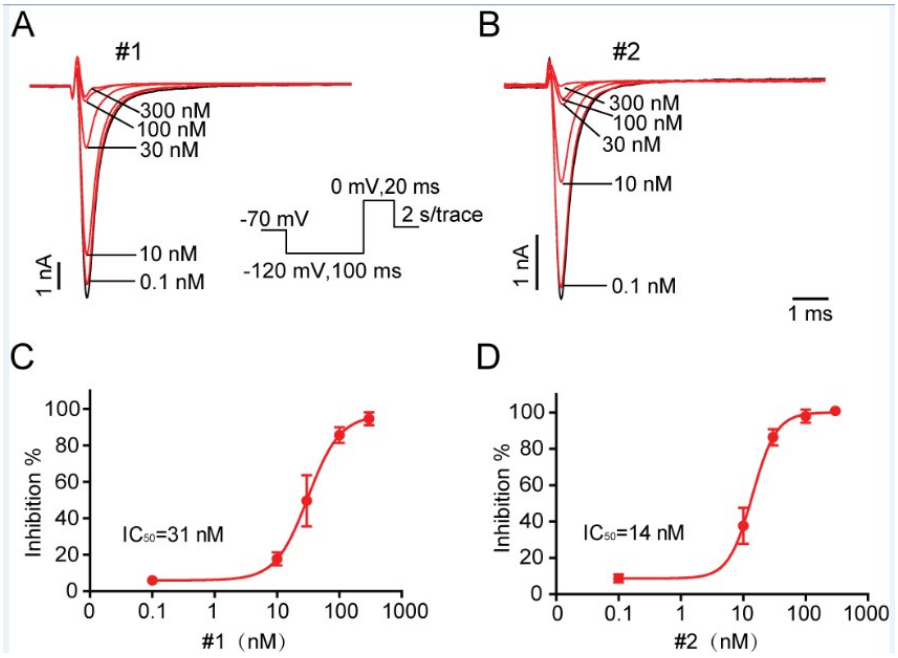


Figure S37. Dose-response curves of GpTx-1 and GpTx-1-71 against hNav1.7

Table S1**. Concentration of GpTx-1 (#1) against hNav1.7**

Concentration	**Inhibition% (mesu±SEM)**	**n**

Control	**2 % ±1 %**	**3**
0.1 nM	**5 % ±2 %**	**3**
10 nM	**18 % ±3 %**	**3**
30 nM	**49 % ±14 %**	**3**
100 nM	**85 % ±4 %**	**3**
300 nM	**95 % ±3 %**	**3**

Table S2**. Concentration of** GpTx-1-71 **(#2) against hNav1.7**

Concentration	**Inhibition% (mesu±SEM)**	**n**
Control	**0.5 % ±1 %**	**3**
0.1 nM	**9% ±2 %**	**3**
10 nM	**38 % ±9 %**	**3**
30 nM	**86 % ±4 %**	**3**
100 nM	**98 % ±3 %**	**3**
300 nM	**100 % ±1 %**	**3**

2.13 Disulfide Bond Analysis (NMR-based analysis)

The structure of GpTx-1 was obtained by high resolution NMR spectroscopy in 90% water and 10% D_2O at pH ~3 and T = 298 K. Homonuclear ^1H-^1H 2D NMR spectra were acquired to determine the structure of GpTx-1. Three NMR spectra were obtained using a Varian 700 MHz spectrometer: DQF-COSY, TOCSY, and NOESY. The 3D structure of GpTx-1 was determined by obtaining the upper distant limits derived from the NOESY spectrum and iterative structure calculation cycles. Most of the backbone and side-chain ^1H resonances were assigned (Figure S38). In total, 457 inter-proton distance restraints obtained by NOESY were used to calculate the structure with Xplor-NIH. The final 20 structures with the lowest energies were selected to represent the structure of GpTx-1 (Figure S39A). The structure of GpTx-1 comprises two antiparallel β-sheets between Leu21-Ser24 and Lys28-Tyr32 (Figure S39B). Furthermore, the NMR structure is

consistent with the expected disulfide bond connectivity of Cys^{2}-Cys^{17}, Cys^{16}-Cys^{30} and Cys^{9}-Cys^{23}.

Method

The resonances were assigned based on three homonuclear spectra, DQF-COSY, TOCSY, and NOESY (mixing time, 300 ms), which were collected on an Agilent 700 MHz spectrometer. The NMR spectra were processed using NMRPipe and analyzed using Sparky. The distance restraints obtained by NOESY were applied to the calculation of the GpTx-1 (457 restraints) and GpTx-1-71 (497 restraints) structures using Xplor-NIH. Two hundred structures were calculated, and the 20 structures with the lowest energies were selected for structure quality assessment. The quality of the structures was assessed using PROCHECK-NMR and MOLMOL.

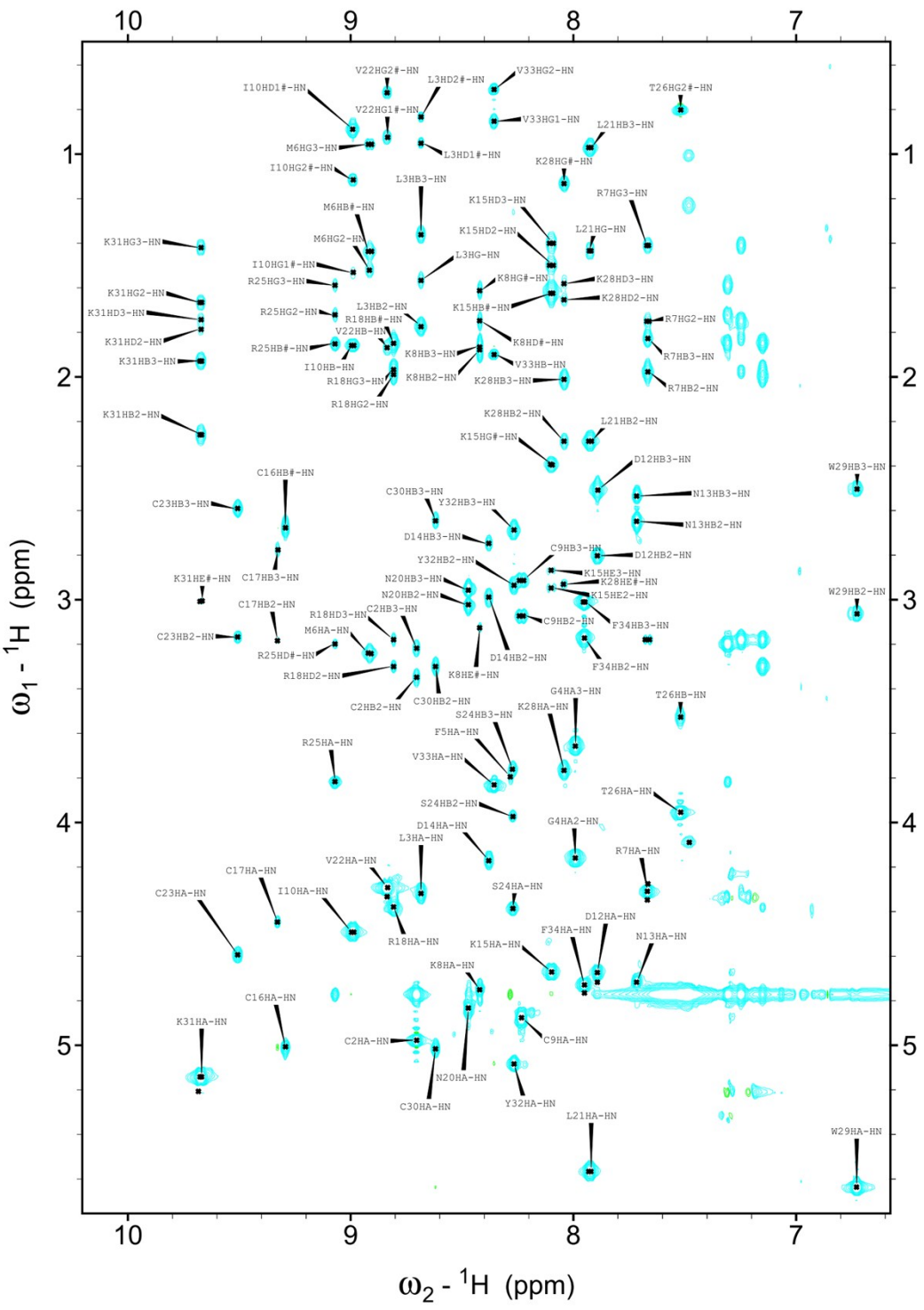


Figure S38 The profile of GpTx-1 proton assignment

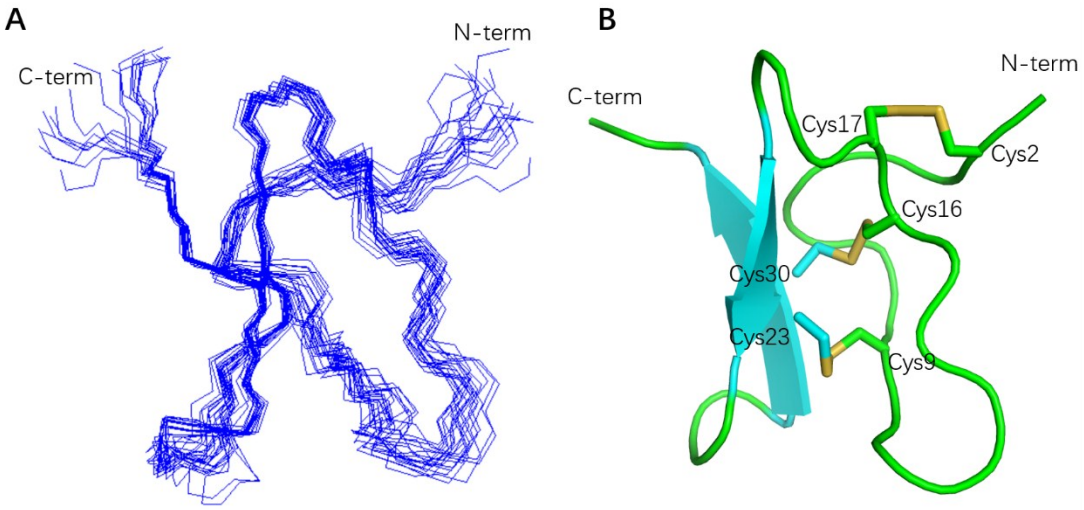


Figure S39 Solution NMR structure of toxin GpTx-1. (**A**) Superposition of the final 20 backbone structures, with the N- and C-termini labeled. (**B**) Cartoon representation of the GpTx-1 structure with three disulfide bonds (yellow sticks).

2.14 IR Spectroscopy

The Fourier Transform Infrared (FTIR) spectrum of GpTx-1 and GpTx-1-71 were recorded using IRPrestige-21 spectrophotometer (Shimadzu). The sample were prepared as a compressed potassium bromide (KBr) pellet. The characteristic bands observed in the FTIR spectrum were summarized in Table 3 and Table 4. The IR spectrum were shown in Figure 32 and Figure S34.

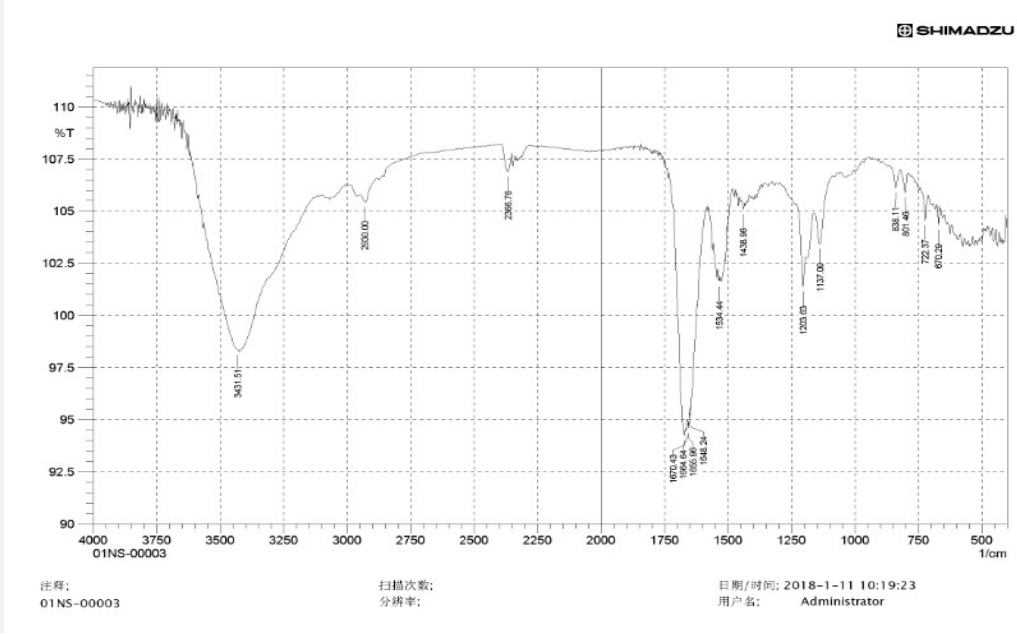


Figure S40. FTIR Spectrum of GpTx-1

Table S3 Assignment of Typical Bands Observed in FTIR Spectrum

Absorption Peak (cm^{-1})	**Type of Vibration**	**Functional Group**
3431.5	O-H stretching	O-H
2930.0	C-H stretching	C-H
1670.4	N-H deformation	N-H
1534.4		
1203.6	C-O stretching	C-O
1137.1		

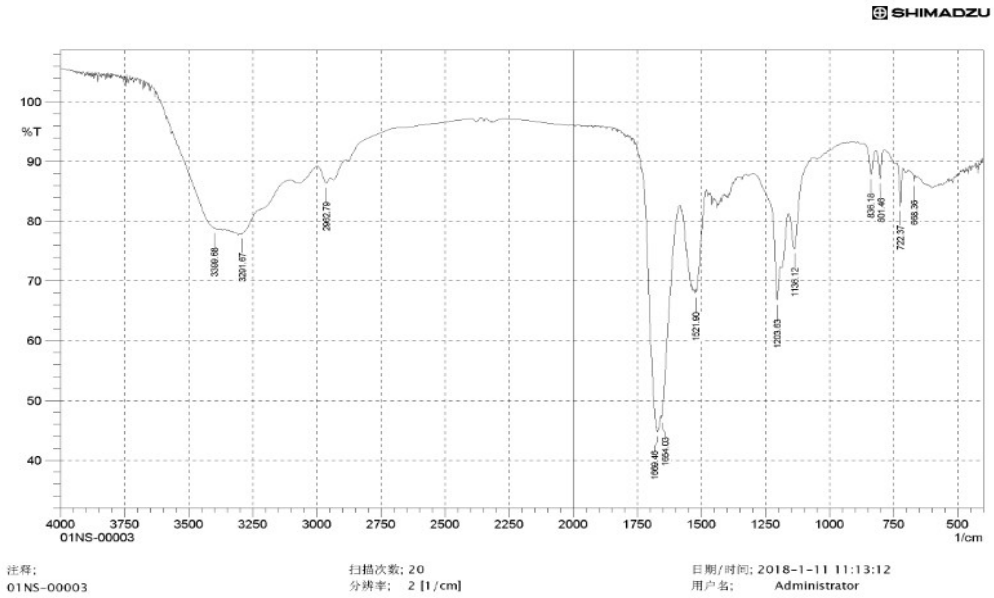


Figure S41. FTIR Spectrum of GpTx-1-71

Table S4. Assignment of Typical Bands Observed in FTIR Spectrum

Absorption Peak (cm^{-1})	**Type of Vibration**	**Functional Group**
3291.7	O-H stretching	O-H
2962.8	C-H stretching	C-H
1669.4	N-H deformation	N-H
1521.9		
1203.6	C-O stretching	C-O

Absorption Peak (cm^{-1})	Type of Vibration	Functional Group
1136.1		

2.15 Circular Dichroism Spectroscopy

Sample of GpTx-1 and GpTx-1-71 were analyzed on Applied photophysics Spectrophotometer. The concentrations GpTx-1 and GpTx-1-71 were 0.06 mg/mL and 0.08 mg/mL respectively (prepared in water).

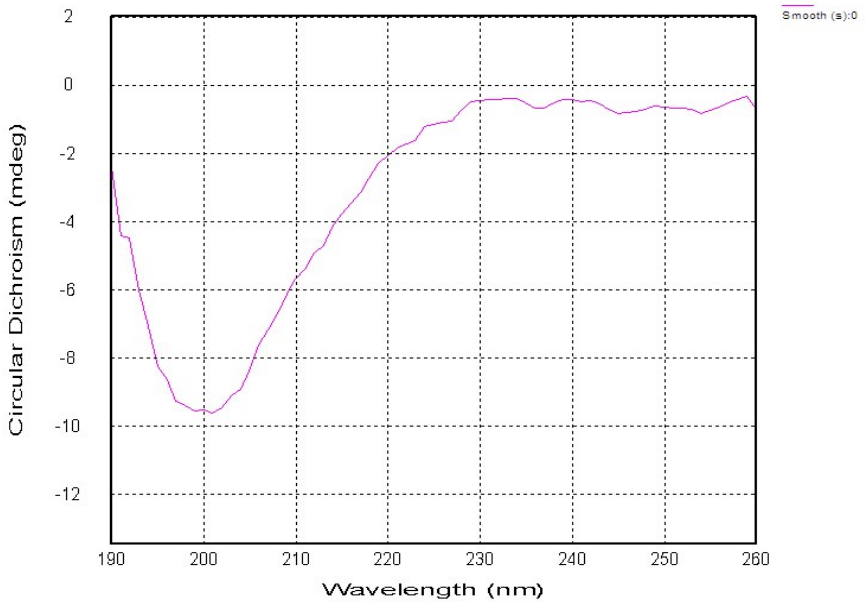


Figure S42. Far UV Circular Dichroism Spectrum of GpTx-1

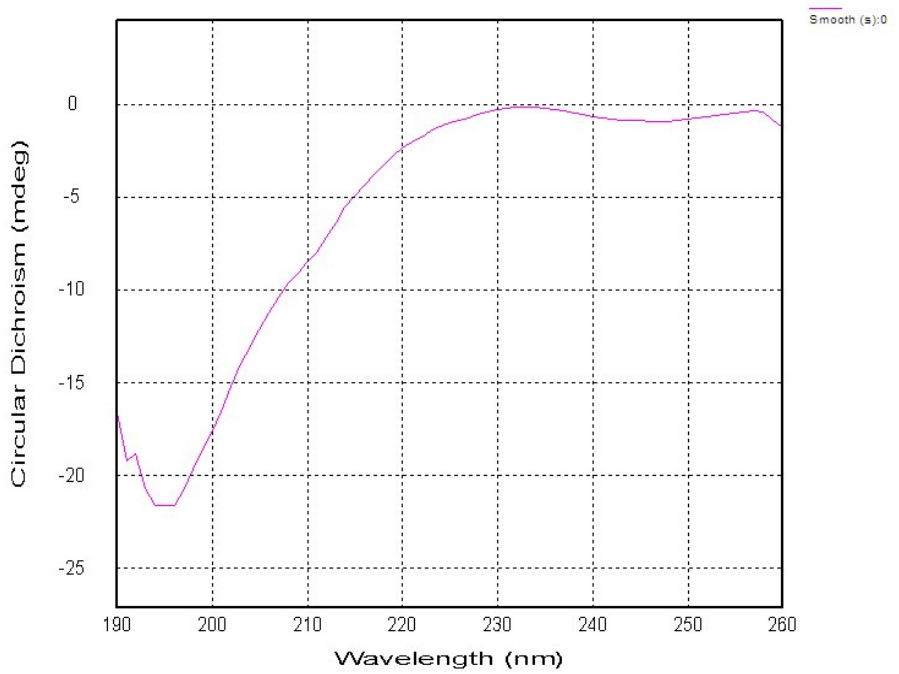


Figure S43. Far UV Circular Dichroism Spectrum of GpTx-1-71

2.16 Optical Rotation

0.6 mg GpTx-1 (TFA salt) and GpTx-1-71 (TFA salt) were dissolved in MeOH (2 mL). The optical rotation tests of GpTx-1 and GpTx-1-71 were performed on Autopol I Polarimeter. $[]^{20}_{D}$= -13 (*C* 0.03, MeOH; GpTx-1); $[]^{20}_{D}$= 20 (*C* 0.03, MeOH; GpTx-1-71).