Supporting information

Design, synthesis and antiproliferative activities of stapled melittin peptides

Ye Wu,[†a] Meng-fei Han,[†b] Chao Liu,[c] Tai-yu Liu,[c] Yu-fei Feng,[c] Yan Zou,[c] Bai Li*[b] and Hong-li Liao*[a]

Email: libai9@126.com; liaohl213@126.com

a) School of Pharmacy, Chengdu Medical College, Chengdu 610083, China

b) Department of Traditional Chinese Medicine, Changhai Hospital, Second Military Medical University, Shanghai 200433, China

c) Department of Organic Chemistry, School of Pharmacy, Second Military Medical University, Shanghai 200433, China.

1. General Information

1.1 Materials

All reagents and solvents were purchased from J&K Scientific, GL Biotech, Energy Chemical or Sinopharm Chemical Reagent Co. Ltd and were purified when necessary. Rink Amide MBHA resin (0.28 mmol/g loading) was purchased from Tianjin Nankai Hecheng Science & Technology Co. Ltd. All other commercially obtained reagents and solvents were used directly without further purification.

1.2 HPLC

Analytical HPLC was run on a SHIMADZU (Prominence LC-20AD) instrument using an analytical column (Grace Vydac "Protein & Peptide C18", 250 × 4.6 mM, 5 μm particle size, flow rate 1.0 mL/min, r.t.). Analytical injections were monitored at 214 nm. Semi preparative HPLC was run on a SHIMADZU (LC-6A) instrument using a semi preparative column (Grace Vydac "Peptide C18", 250 × 10 mM, 10 μm particle size, flow rate 4 mL/min). Solution A was 0.1% TFA in water, and solution B was 0.1% TFA in MeCN. Gradient A: A linear gradient of 10% to 10% B over 2 mins, then a linear gradient of 10% to 90% B over 25 mins. Gradient B: A linear gradient of 1% to 1% B over 3 mins, then a linear gradient of 1% to 35% B over 25 mins.

2. Peptide synthesis

As a typical example, 400 mg Rink Amide MBHA resin was swelled with DCM (5 mL) for 20 mins. Then the resin was treated with 20% piperidine/DMF twice (10 and 5 mins), followed by washing with DMF (5 times), DCM (5 times) and DMF (5 times). For coupling of the first amino acid, Fmoc-AA-OH (1 mmol), HCTU (0.9 mmol), DIEA (2 mmol) and DMF (6 mL) were mixed for 2 mins and then added to the resin. After 2 h, the resin was washed with DMF (5 times), DCM (5 times), and DMF (5 times). The peptide couplings of S_5/R_8 were carried out over a single two hour coupling cycle using 2 eq. of the Fmoc protected amino acids. The deprotection, washing, coupling and washing steps were repeated until all the amino acid residues were assembled. The peptide-bound resin was treated with 20% piperidine/DMF to remove the Fmoc group from the N-terminus. After the resin was washed it was treated with 3 mL solution of

acetic anhydride and pyridine (1:1) for 20 mins. Then the resin was washed with DMF (5 times), DCM (5 times), and DMF (5 times). The ring-closing metathesis reaction was carried out in 1,2-dichloroethane (DCE) at 35 °C using Grubbs' first-generation catalyst, The resin was washed with DCM (3 times), and DCE (3 times) and then treated with 10 mM solution of Grubbs' first-generation catalyst in DCE. After the first round of the 2 h metathesis, we repeated the same procedure for a second round of catalyst treatment with fresh catalyst solution, the peptide-resin was washed with DMF (5 times), DCM (5 times). Peptides were cleaved from their resin by treatment with reagent K (80% TFA, 5%, H_2O, 2.5% EDT, 5% Thioanisole and 7.5% phenol) for 4 hrs at room temperature. After completion of the cleavage reaction, TFA was evaporated by blowing with Ar. The crude peptides were obtained by precipitation with 40 mL of cold diethyl ether and centrifugation at 3500 r/min for 3 mins (3 times). The supernatant diethyl ether was decanted from the centrifuge tube and the crude peptides were allowed to air dry.

Table S1. Electrospray MS data for peptides (positive mode)

Peptide	Molecular Weight (g/mol)	Found Mass
Melittin	2844.75	$[M+3H]^{3+}$ = 949.5960; $[M+4H]^{4+}$ = 712.4511
Mel-S1	2880.79	$[M+3H]^{3+}$ = 961.6079; $[M+4H]^{4+}$ = 721.4584
Mel-S2	2978.90	$[M+3H]^{3+}$ = 994.3110; $[M+4H]^{4+}$ = 745.9859
Mel-S3	2980.88	$[M+3H]^{3+}$ = 994.9714; $[M+4H]^{4+}$ = 746.4806
Mel-S4	2909.83	$[M+3H]^{3+}$ = 971.2882; $[M+4H]^{4+}$ = 728.7187; $[M+5H]^{5+}$ = 583.1776
Mel-S5	2184.38	$[M+2H]^{2+}$ = 1093.6992; $[M+3H]^{3+}$ = 729.4699

3. Circular dichroism

The linear and stapled peptides were dissolved in the phosphate buffer solutions (10 mM, pH = 7.4) to a final concentration of 50 μM, respectively. The CD spectra were obtained with 1 mm quartz cuvette on Jasco-715 spectropolarimeter at 25 ^{o}C. The measurement parameters were set up as follows: wavelength, 185-260 nm; step resolution, 0.5 nm; speed, 20 nm min^{-1}; accumulation, 3. All spectra were converted to a uniform scale of molar ellipticity after background subtraction. The curves were smoothed using standard parameters. In addition, according to the ellipticity of the

peptide's spectrum at 222 nm and the number of amino acids in the peptide sequence, we calculated the helicity of each peptide using the literature equation[1]

4. Anti-hepatoma assay

Anti-hepatoma activity was evaluated by the standard MTT assay with a slight modification measuring the median inhibitory concentration (IC_{50}) values.[2] SMMC-7721 cells and HepG2 cells were plated in triplicate wells into a 96-well plate at 5×10^3/well and cultured routinely for 24 h. Cells were infected with peptides and continued to culture with DMEM plus 10% FBS at 37 °C and 5% CO_2. After being incubated for 48, 72 and 96 h, the absorbance A value was determined by MTT at 490 nm. The untreated SMMC-7721 cells and HepG2 cells were used as control. The inhibitory rate was calculated according to the following formula: Inhibitory rate = (A value of the control group – A value of the study group) / A value of the control group × 100%.

5. Hemolysis assay

The hemolytic activity of the peptides was evaluated by determining hemoglobin release from erythrocyte suspensions of fresh rabbit blood.[3] The cells were centrifuged, washed three times with physiological saline (NS), dispensed into 96-well plates as 200 μL of 4 % (v/v) red blood cells in NS, and different peptide solutions were added to each well. Plates were incubated for 1 h at 37 °C, then centrifuged at 1,000 rpm for 10 min. Samples (100 μL) of supernatant were transferred to 96-well plates and hemoglobin release was monitored by measuring absorbance at 450 nm. Zero hemolysis was determined in NS (ANS) and 100 % hemolysis was determined in 0.1 % (v/v) Triton X-100 (ATriton). Hemolysis percentage hemolysis was calculated as: $(A_{peptide} - A_{NS})/(A_{Triton} - A_{NS}) \times 100$ %. All hemolysis determinations were conducted in three repeated experiments.

6. *α*-Chymotrypsin digestion assay.

The peptides were dissolved in PBS buffer solutions (50 mM, pH = 7.4) to a final concentration of 1mM, respectively. *α*-Chymotrypsin was dissolved in PBS buffer (50 mM, containing 2 mM of $CaCl_2$, pH = 7.4) to a final concentration of 5 ng/μL. Then

the peptide solutions (100 μL) were incubated with *α*-Chymotrypsin solution (1 mL) at room temperature. 100 μL of digestion mixture was taken at the 0, 5, 10, 15, 20, 30, 40, and 60 min marks, then quenched with 20 μL of hydrochloric acid (1 M). The solution of the *α*-Chymotrypsin peptide fragments was monitored by HPLC at different times to determine the fraction of protease degradation at 214 nm. Each experiment was performed in duplicate.

7. Spectrum

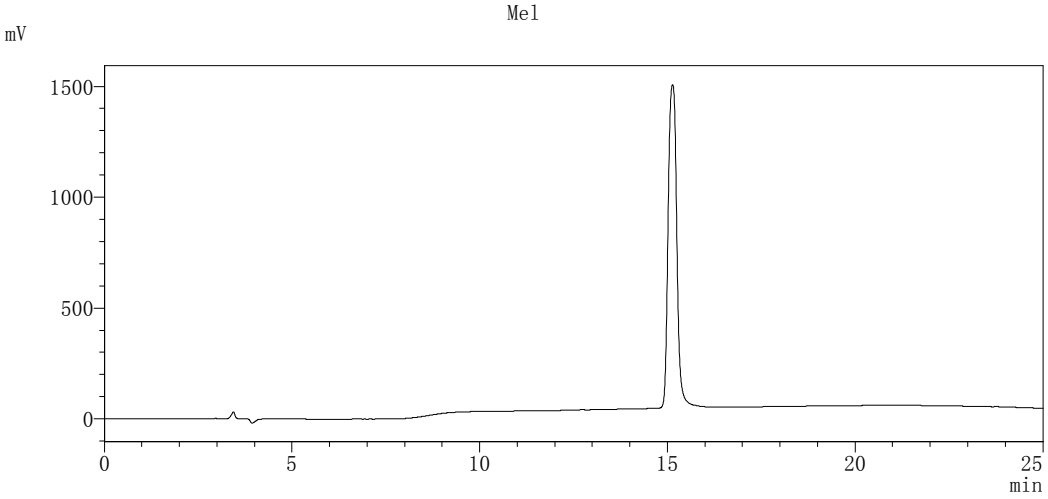

Figure S1. Analytical HPLC trace for compound **Melittin**

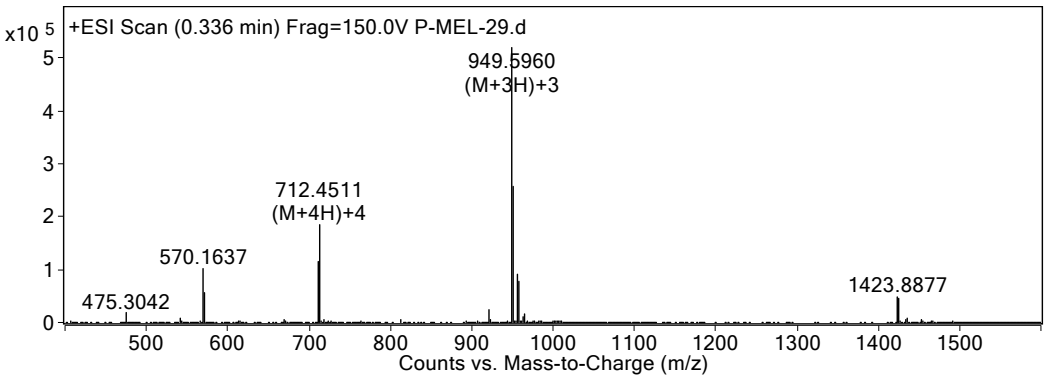

Figure S2. HR-Q-TOF-MS for compound **Melittin**

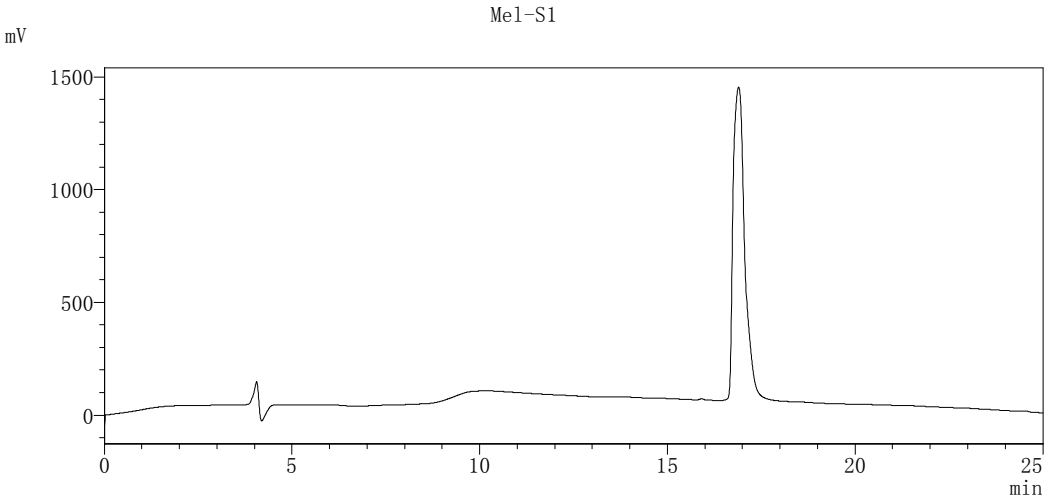

Figure S3. Analytical HPLC trace for compound **Mel-S1**

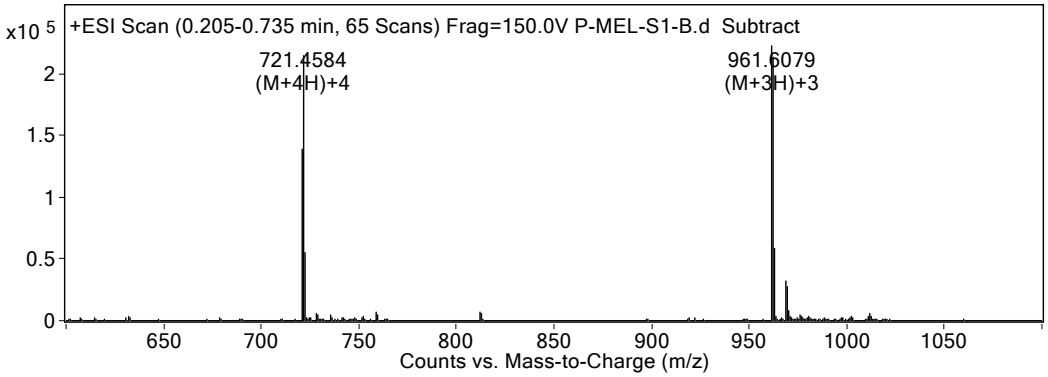

Figure S4. HR-Q-TOF-MS for compound **Mel-S1**

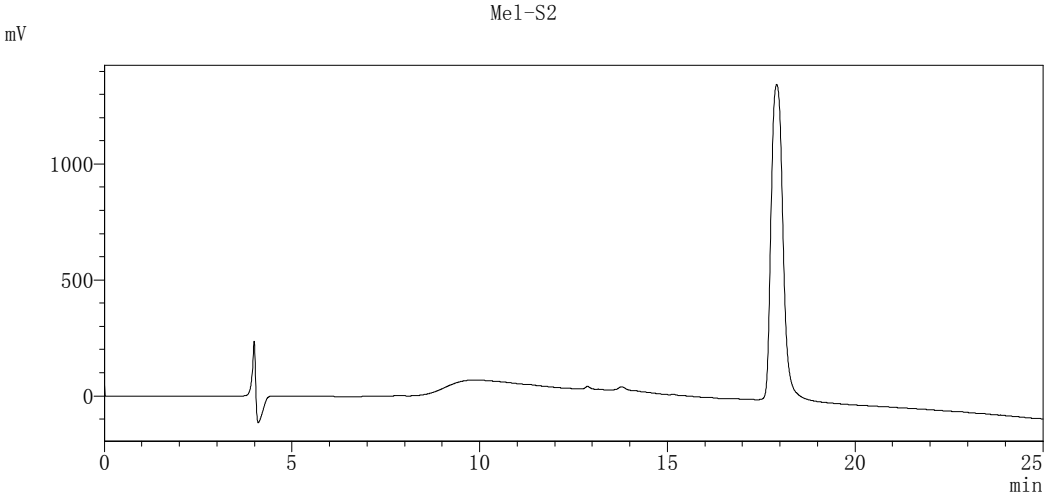

Figure S5. Analytical HPLC trace for compound **Mel-S2**

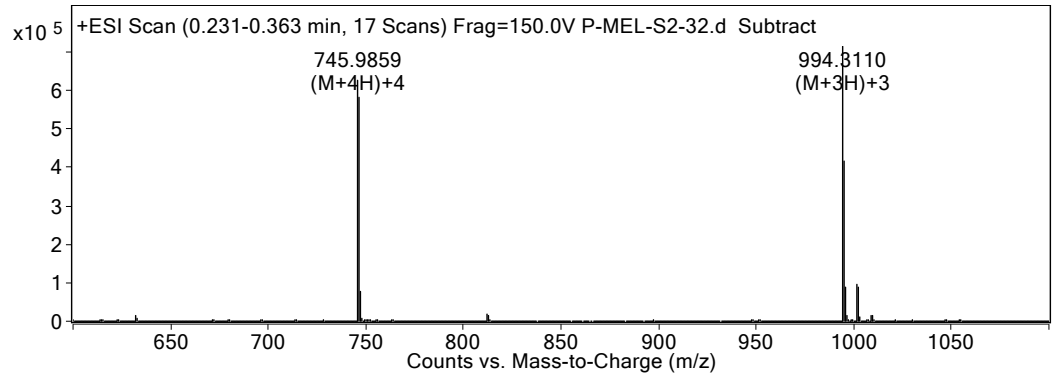

Figure S6. HR-Q-TOF-MS for compound **Mel-S2**

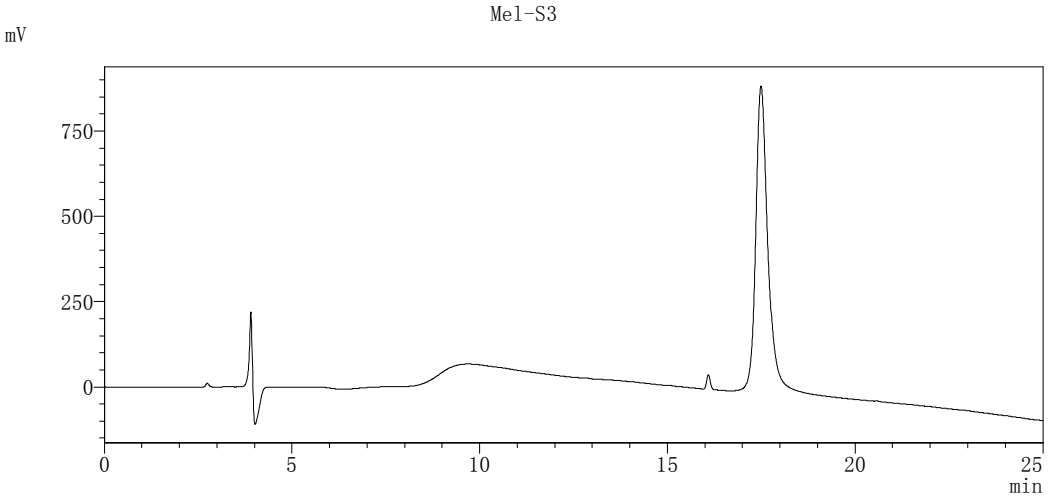

Figure S7. Analytical HPLC trace for compound **Mel-S3**

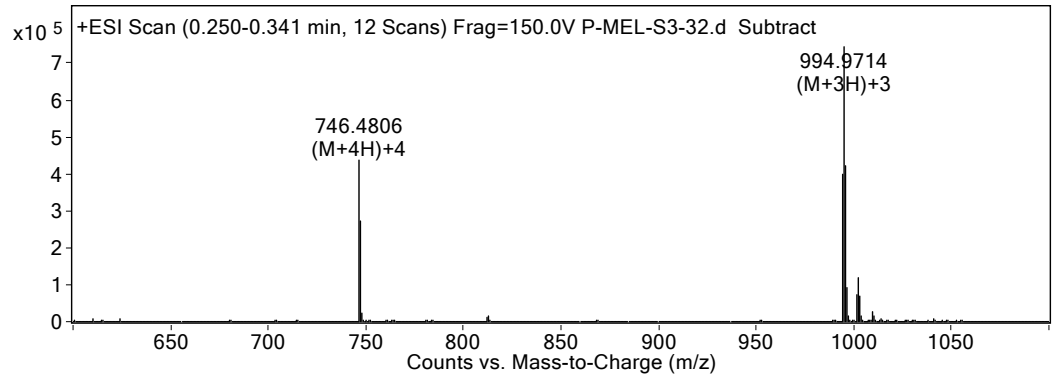

Figure S8. HR-Q-TOF-MS for compound **Mel-S3**

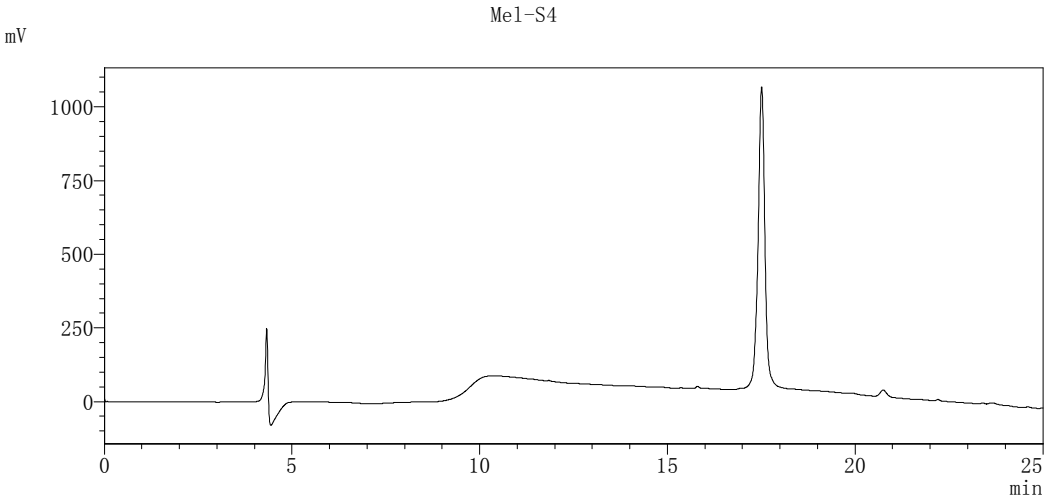

Figure S9. Analytical HPLC trace for compound **Mel-S4**

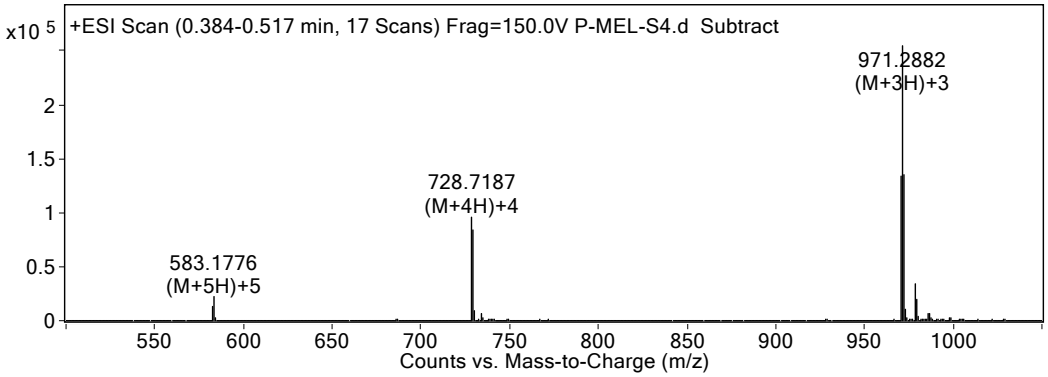

Figure S10. HR-Q-TOF-MS for compound **Mel-S4**

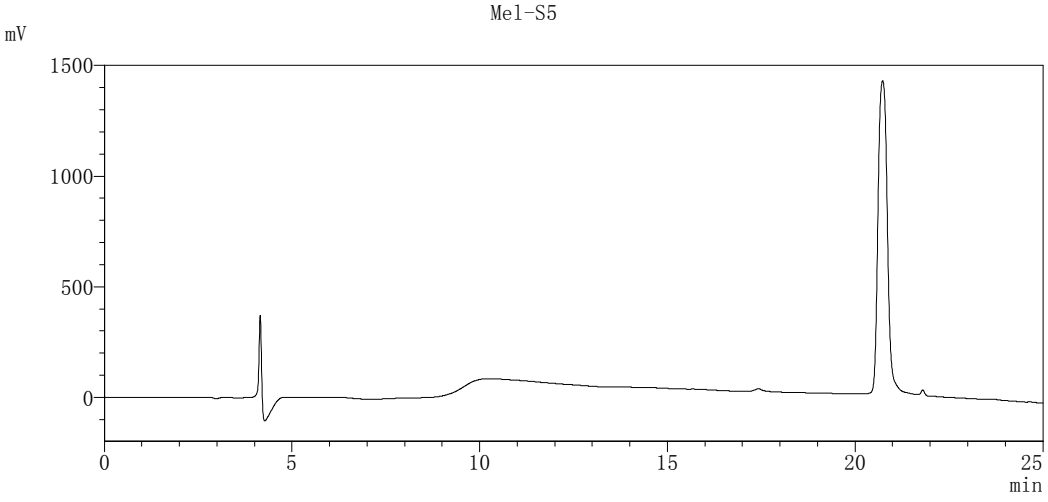

Figure S11. Analytical HPLC trace for compound **Mel-S5**

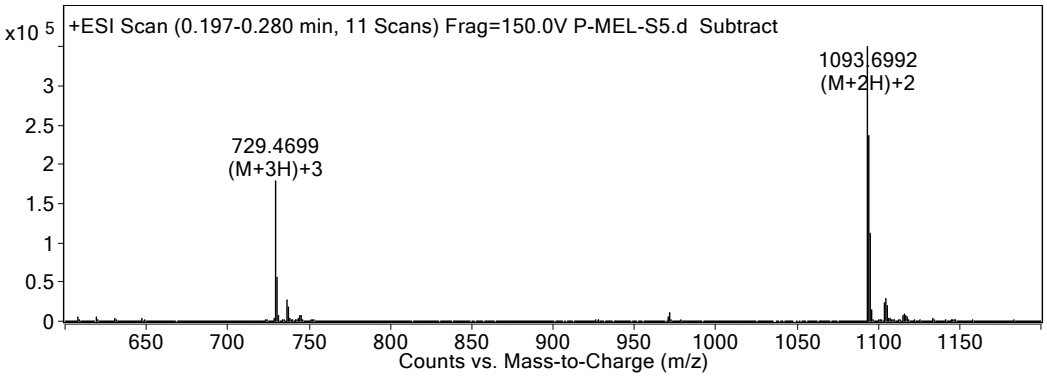

Figure S12. HR-Q-TOF-MS for compound **Mel-S5**

8. References

1. H. K. Cui, J. Qing, Y. Guo, Y. J. Wang, L. J. Cui, T. H. He, L. Zhang and L. Liu, *Bioorg. Med. Chem.*, 2013, **21**, 3547-3554.
2. B. Li, W. Gu, C. Zhang, X. Q. Huang, K. Q. Han and C. Q. Ling, *Onkologie*, 2006, **29**, 367-371.
3. H. Liu, Y. Han, H. Fu, M. Liu, J. Wu, X. Chen, S. Zhang and Y. Chen, *Appl. Microbiol. Biot.*, 2013, **97**, 2877-2884.