SUPPORTING INFORMATION

Asteropsins B-D, Sponge-Derived Knottins with Potential Utility as a Novel Scaffold for Oral Peptide Drugs

Huayue Li[a], John J. Bowling[b,e], Mingzhi Su[a], Jongki Hong[c], Bong-Jin Lee[d], Mark T. Hamann[b,**], Jee H. Jung[a,*]

[a] College of Pharmacy, Pusan National University, Busan 609-735, Korea

[b] Department of Pharmacognosy, School of Pharmacy, The University of Mississippi, Oxford, Mississippi 38677, USA

[c] College of Pharmacy, Kyung Hee University, Seoul 130-701, Korea

[d] College of Pharmacy, Seoul National University, Seoul 151-742, Korea

[e] Present address: Department of Chemical Biology and Therapeutics, St. Jude Children's Research Hospital, Memphis, Tennessee 38105, USA

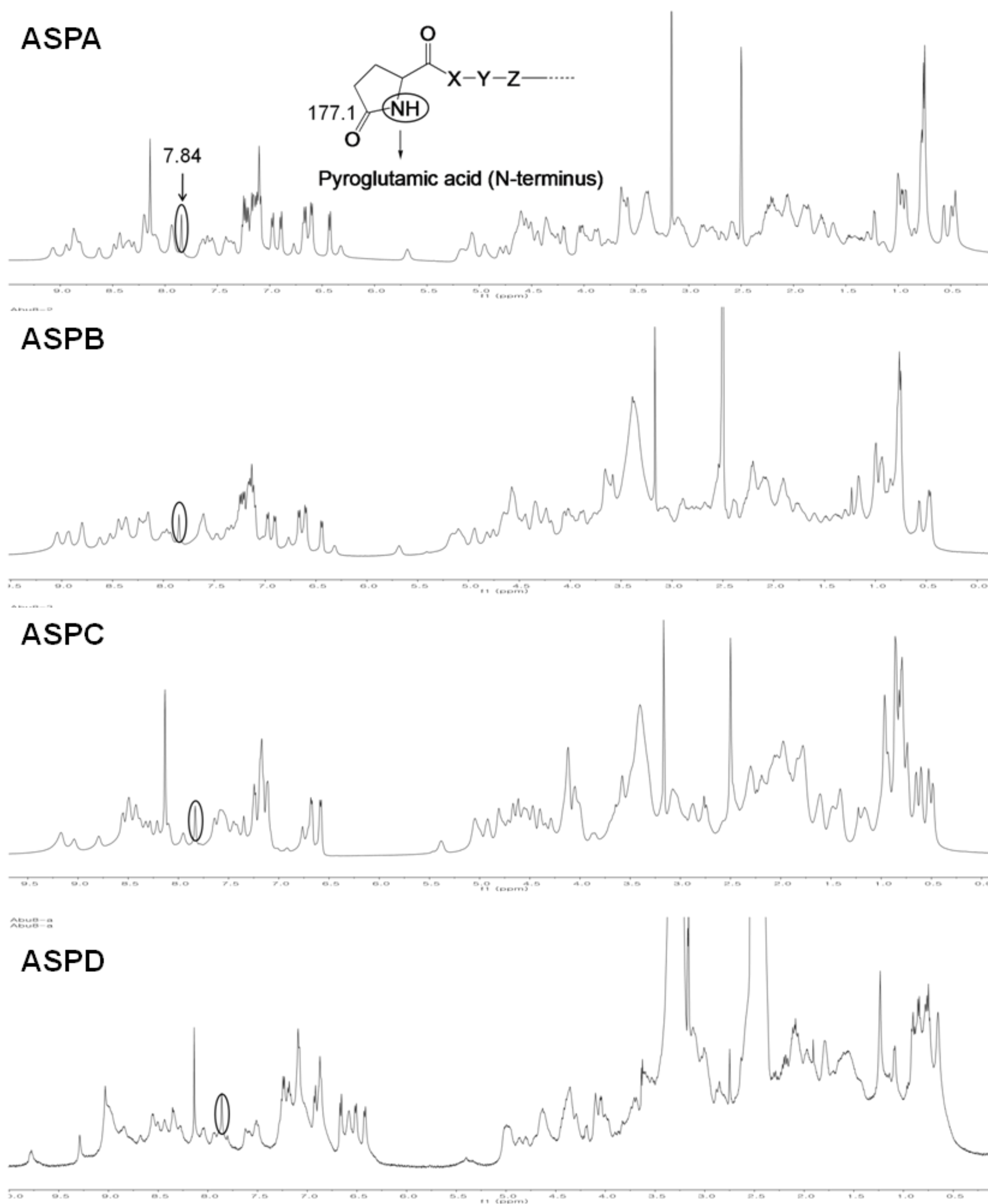

Fig. S1. ^{1}H NMR spectra of Asteropsins A-D (ASPA, ASPB, ASPC, and ASPD) in DMSO-d_6 (500 MHz). The circled sharp peaks correspond to the amide proton of *N*-terminal pyroglutamyl ring.

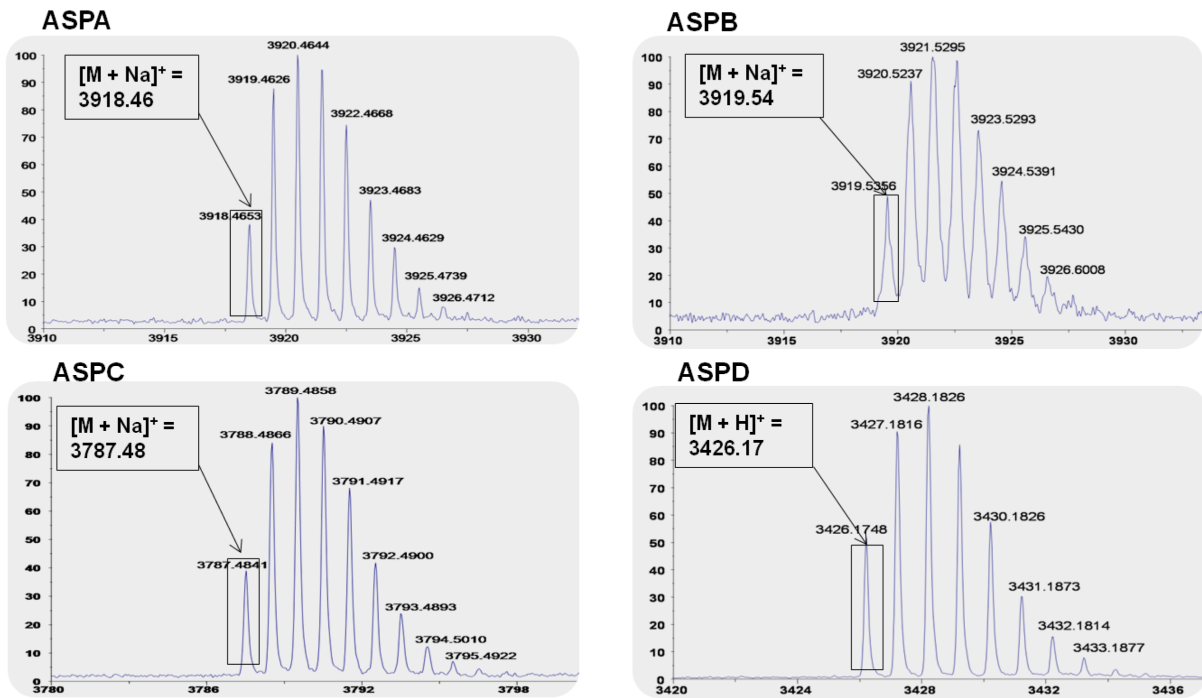

Fig. S2. MALDI-TOF MS spectra of Asteropsins A-D.

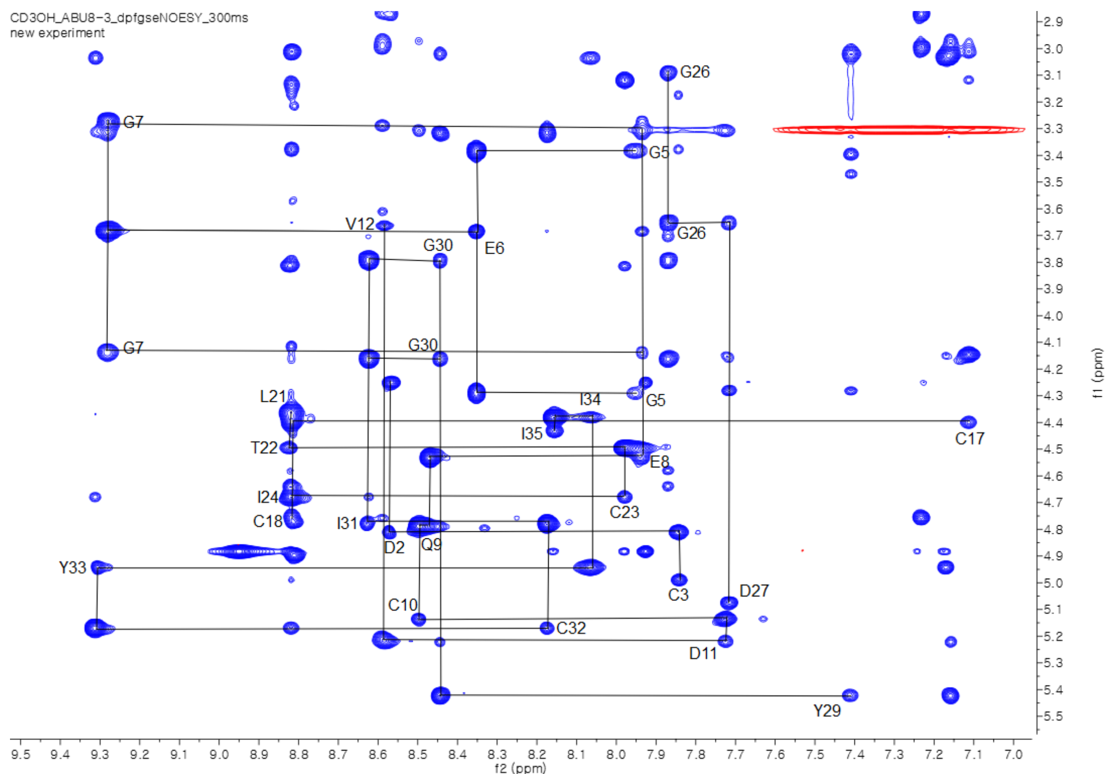

Fig. S3. Sequential C$^{\alpha}$H(i)-NH(i + 1) connectivities of ASPC in the C$^{\alpha}$H-NH fingerprint region of the NOESY spectrum recorded in CD_3OH (900 MHz). The mixing time of the NOESY spectrum is 300 ms. Intra-residue C$^{\alpha}$H-NH cross peaks are marked with the one-letter code for the amino acid and residue number.

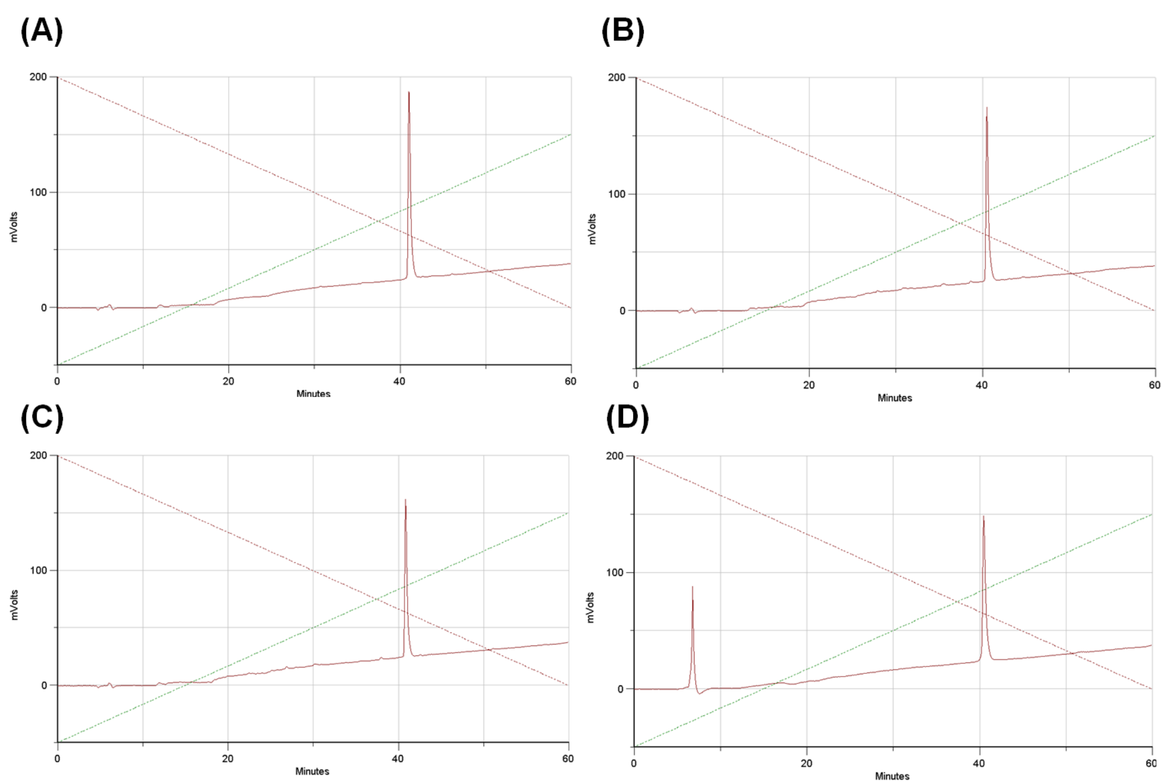

Fig. S4. HPLC chromatograms of remaining ASPA after enzymatic degradation by thermolysin and proteinase K. (A) Standard ASPA (B) ASPA + thermolysin, 4 h (C) ASPA + thermolysin, 12 h, (D) ASPA + proteinase K, 48 h. According to the peak area of HPLC chromatograms, the remaining amount of ASPA was (B) 91%, (C) 80%, and (D) 94% compared to (A) the beginning point.

Table S1

Analysis of the possible disulfide bonding patterns in ASPB and ASPC.

Disulfide pattern	Mean distance (Å)[a]	
	ASPB	ASPC
I-II	9.9 ± 0.4	9.6 ± 0.8
I-III	9.0 ± 1.4	8.8 ± 0.4
I-IV	**4.3 ± 0.9**	**4.4 ± 1.2**
I-V	11.7 ± 2.2	12.5 ± 0.4
I-VI	9.3 ± 0.7	8.9 ± 0.4
II-III	6.1 ± 0.9	4.5 ± 1.3
II-IV	10.9 ± 0.8	9.3 ± 1.2
II-V	**4.2 ± 0.6**	**4.2 ± 1.1**
II-VI	4.2 ± 1.0	4.4 ± 0.2
III-IV	8.2 ± 0.6	7.7 ± 1.0
III-V	5.5 ± 2.3	6.0 ± 2.1
III-VI	**4.4 ± 1.2**	**4.5 ± 1.1**
IV-V	11.4 ± 1.1	11.7 ± 0.8
IV-VI	9.1 ± 0.2	7.4 ± 1.0
V-VI	5.7 ± 1.5	6.3 ± 0.1

[a] Mean S-S distances (Å) calculated from the 20 lowest energy structures without disulfide bond restraints

Table S2

Cytotoxicity of asteropsins A-D[a] against human solid tumor cell lines.

Peptide	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
ASPA	> 30	> 30	> 30	> 30	> 30
ASPB	> 30	> 30	> 30	> 30	> 30
ASPC	> 30	> 30	> 30	> 30	> 30
ASPD	> 30	> 30	> 30	> 30	> 30
Doxorubicin	0.0012	0.0352	0.0011	0.0304	0.0475

[a] Data expressed in ED_{50} values (μg/mL). A-549, human lung cancer; SK-OV-3, human ovarian cancer; SK-MEL-2, human skin cancer; XF-498, human CNS cancer; HCT-15, human colon cancer.

SUPPLEMENTARY EXPERIMENTAL METHODS

Trypsin and chymotrypsin inhibitory assay. The enzymatic activity of bovine trypsin and chymotrypsin (Sigma-Aldrich) was measured spectrophotometrically. Trypsin was dissolved in 50 mM Tris/HCl, pH 8.0, to a final concentration of 20 mg/mL. Chymotrypsin, was dissolved in 10 mM Tris/HCl, pH 8.0, to a final concentration of 1 μg/mL. Enzymes were incubated for 10 min at 37°C in the presence of various concentrations of peptides (0.01 – 100 μg/mL). After incubation, the remaining enzymatic activity was determined using the corresponding chromogenic substrates: BApNA (N_{α}-benzoyl-DL-arginine *p*-nitroanilide) for trypsin and *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide for chymotrypsin. Final concentration of the substrates was 0.5 mg/mL. Kinetics of *p*-nitroaniline release was measured at 405 nm.

Enzymatic degradation by thermolysin and proteinase K. Peptides, thermolysin (from *Bacillus thermoprotelyticus* Rokko), and proteinase K (from *Tritirachium album*) were prepared in 50 mM Tris-HCl buffer (pH 8.0). The peptide solution (0.5 mg/mL, 225 μL) were added to 25 μL of thermolysin (4 mg/mL) and proteinase K (2 mg/mL), respectively. Digestions using thermolysin was incubated at 65°C and proteinase K at 50°C. Aliquots (20 μL) were taken at appropriate time intervals and was analyzed by reversed-phase HPLC using a UV detector (YMC ODS column 250 mm × 4.6 mm, i.d. 5 μm; wavelength: 220 nm) at a flow rate of 0.5 mL/min with a linear gradient elution (0-80% solvent B; solvent A: H_2O + 0.1% TFA, solvent B: 90% ACN + 0.1% TFA).

Cytotoxicity against human cancer cell lines. Rapidly growing cells (A-549, human lung cancer; SK-OV-3, human ovarian cancer; SK-MEL-2, human skin cancer; XF-498, human CNS cancer; HCT-15, human colon cancer) were harvested, counted, and inoculated at the appropriate concentrations ((1-2) × 10^4 cells/ well) into 96-well microtiter plates. After incubation for 24 h, the compounds dissolved in culture medium (RPMI 1640, Gibco; 10% FBS, Gibco) were applied to the culture wells in triplicate followed by incubation for 48 h at 37°C under a 5% CO_2 atmosphere. The culture was fixed with cold TCA, and was stained by 0.4% SRB (sulforhodamine B, Sigma) dissolved in 1% acetic acid. After solubilizing the bound dye with 10 mM unbuffered Tris base using a gyrotatory shaker, the absorbance at 520 nm was measured with a microplate reader (Dynatech Model MR 700). The fifty percent inhibitory concentration (ED_{50}) was defined as the concentration that reduced absorbance by 50% compared to the control level in untreated wells.