Insecticidal Peptide from Mungbean: A Resistant Factor against Infestation with Azuki Bean Weevil

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A resistant factor from a strain (TC1966) of wild mungbean (*Vigna radiata* var. *sublobata*) active against the azuki bean weevil (*Callosobruchus chinensis*) was genetically transferred into a susceptible cultivar. The resulting strain ($BC_{20}F_4$) tolerated an infestation by *C. chinensis*. The ethanol extract of $BC_{20}F_4$ inhibited larval growth. Vignatic acid A (**1**) was isolated and evaluated as one of the inhibitors present in $BC_{20}F_4$. Structure **1** was determined to be a cyclopeptide alkaloid composed of L-tyrosine, 3(*S*)-hydroxyl-L-leucine, L-phenylalanine, and 2-hydroxyisocaproic acid by FAB-HRMS, DQF-FGCOSY, FG-HMQC, FG-HMBC, and NOE-DF. This compound is the first example of an insecticidal cyclopeptide alkaloid base of plant origin.

Keywords: *Mungbean; Vigna radiata; azuki bean weevil; Callosobruchus chinensis; azuki bean; Vigna angularis; vignatic acid; cyclopeptide alkaloid*

INTRODUCTION

Seeds of grain legumes in the genus *Vigna*, such as cowpea (*Vigna unguiculata*), mungbean (*Vigna radiata*), and azuki bean (*Vigna angularis*), are staple foods in many countries. The postharvest damage of the seeds caused by a group of bruchids (Coleoptera Bruchidae) is quiet extensive. A wild mungbean strain (TC1966) was found to possess complete resistance to the infestation by four species of major bruchid pests (Fujii et al., 1989). Conventional genetic analysis indicated that the bruchid resistance of TC1966 was controlled by a single dominant locus (Kitamura et al., 1988), but the product of this locus has never been characterized. Here we report a cyclopeptide alkaloid, vignatic acid A, as one of the inhibitory factors conferring the bruchid resistance. This compound is the first example among the cyclopeptide alkaloids in plant that has insecticidal activity.

A resistant gene (*R*) of the wild strain TC1966 was hybridized into a susceptible cultivar (Osaka-ryokuto) to give a $BC_{20}F_4$ resistant isogenic line (IL) by the selection for bruchid resistance against azuki bean weevil (*Callosobruchus chinensis*) (Kitamura et al., 1988; Ishimoto and Kitamura, 1993). The lipid contents in the seed flour were removed by acetone, and then the residue was extracted with 80% ethanol. After centrifugation and removal of the pellet, the supernatant was evaporated followed by extraction with 1-butanol. Two peaks denoted vignatic acid A (**1**) and B (**2**) were recognized to be the characteristic chemical differences between resistant and susceptible seed extracts by comparing their HPLC chromatograms (Figure 1). In bioassays using an artificial bean method (Ishimoto and Kitamura, 1989), the larvae of *C. chinensis* died as first instars just after digging into the artificial beans containing 0.5−1% of vignatic acid A (**1**) in the seed flour of the susceptible variety.

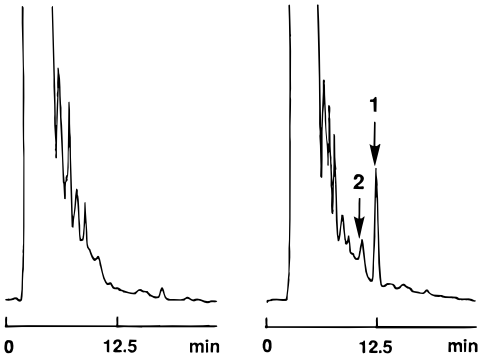

Figure 1. HPLC chromatograms of the extracts of susceptible (left) and resistant (right) strains of mungbeans. Peaks 1 and 2 in the right chromatogram correspond to vignatic acid A and B, respectively.

MATERIALS AND METHODS

Extraction. The flour (10 g) of the mungbean ($BC_{20}F_4$) seeds was washed with acetone to remove the acetone soluble fraction. After filtration, the residue was extracted with 80% EtOH (200 mL) at 5 °C overnight. The extract was centrifuged at 12000*g* for 20 min at 0 °C. The supernatant was collected and evaporated under vacuum at 30 °C to yield a solid material. The solid was dispersed in water (10 mL), and then 1-butanol (10 mL) was added. After centrifugation at 3600*g* for 2 min at 0 °C, the 1-butanol fraction was collected and dried *in vacuo*. The residual solid was then dissolved with 80% EtOH. The flour of Osaka-ryokutou seeds, a susceptible mungbean, was independently extracted for comparison by HPLC with the extract of resistant seeds. The purification was performed on a HPLC equipped with a reverse phase column (7.8 × 300 mm, TOSOH TSKgel ODS 80T_M, Tokyo) eluted with 45% acetonitrile containing 0.1% acetic acid. The eluent was monitored at 225 nm with a flow rate of 2 mL/min.

Amino Acid Analyses. A Hitachi model 835 amino acid analyzer equipped with ion-exchange resin (no. 2617, Hitachi

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Ltd., Tokyo) in a 4 × 250 mm column eluted with Na citric acid buffer (0.3 mL/min) was employed. Each sample solution (50 μL, 100 nmol/mL of **1** and 150 nmol/mL **2**) in 6 N HCl (100 μL) was heated at 110 °C for 72 h in sealed tubes. The resulting solutions were diluted with water (100 μL) and citric acid buffer (pH 2.2, 250 μL) for amino acid analyses. Phenylalanine (80 nmol/mL) from **1** and leucine (120 nmol/mL) from **2** were identified.

D- and L-Amino Acid Analyses. A Hitachi model 835 amino acid analyzer equipped with Senshu Pak PEGASIL ODS (Senshu Scientific Co., Tokyo) in a 6 × 250 mm column eluted with a gradient solvent system (0.1 M acetate buffer, CH_3CN, THF) was employed. The hydrolyzed solution (2.5 μL) of each sample was treated with a solution of (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) (10 mM) in a 0.1 M Na borate buffer (pH 9.0). Peaks were detected with fluorescence with ex 260 nm and em 315 nm. L-Phenylalanine from **1** and L-leucine from **2** were identified by comparing their retention times with those of authentic standards.

Insects. A laboratory colony of *C. chinensis* was obtained from the National Institute of Agro-Environmental Sciences, Tsukuba, Ibaraki 305, Japan, and maintained on azuki bean (*V. angularis* cv. Beni-dainagon) at 30 °C and about 70% relative humidity.

Feeding Tests. The effect of vignatic acid A and B on bruchid development was examined with a feeding test using artificial beans made of susceptible mungbean flour and various concentrations of the compounds. The lyophilized material added to mungbean flour was thoroughly mixed in a rotary mixer to ensure a uniform distribution, and 0.34 mL of distilled water was added to 1 g of the mixture. The mixture formed a columnar shape and then was lyophilized. The artificial beans were weighed and then coated with a 8% gelatin solution. Each treatment was conducted with six replications containing an artificial bean weighing approximately 0.5 g. An artificial bean was placed in a plastic Petri dish into which newly emerged adults were introduced. The plastic dishes were kept in an incubator at 30 °C and about 70% relative humidity. After 24 h, the adults were removed. Seven days after initial oviposition, the number of eggs hatched on the surface of the artificial beans was counted. After 30 days the artificial beans were dissected, and the number of adults, living larvae, and pupae was recorded. Each treatment was conducted with three replications.

Spectral Procedures. NMR measurements were performed on a JEOL JNM-A 600 or JNM-A 400 spectrometer equipped with a pulsed field gradient (PFG) unit. Abbreviations for NMR spectra are as follows: FG-DQFCOSY, field gradient double-quantum frequent correlation spectroscopy; PFG-HMQC, phase-sensitive field gradient heteronuclear multiple-quantum coherence spectroscopy; FG-HMBC, field gradient heteronuclear multiple-bond correlation; NOE-DF, differential nuclear Overhauser effect; and NOEs, nuclear Overhauser effects.

NMR spectra were recorded in DMSO-d_6 or CD_3OD solutions at 600.05 MHz for ^1H and at 100.4 MHz for ^{13}C, and the chemical shifts are given relative to TMS as an internal standard. NOE difference spectra were recorded using a standard pulse sequence with a relaxation delay of 3 s. All 2D spectra were recorded using the standard pulse sequence with *z*-axis PFG. The PFG-DQFCOSY spectra were obtained using a data set of 1K × 1K points with 512 increments with eight transients at magnitude mode. Sine bell-shaped gradient pulses were used with 2:1:4 ratio and 1 ms duration, and maximum strength was 25.6 G/cm. The PFG-HMQC spectra were recorded using a 512 × 1K data set with 16 scans/increment, using a 25.6 G/cm gradient strength for maximum effect. The PFG-HMBC experiments were performed using the same data set and the PFG strength of PFG-HMQC experiments, with 64 scans/increment. For long range C−H coupling, a 60 ms delay time was used.

FAB (fast atom bombardment) mass spectra were run in a glycerol matrix. These spectra, as well as HR (high-resolution)-FAB mass spectra, were conducted using a JEOL JMS-AX505WA mass spectrometer. Specific rotations were recorded on a JASCO DIP-370 digital polarimeter.

Vignatic acid A (1): white powder; t_R 12.5 min; $[\alpha]_D$ −99° (*c* 0.11, MeOH); FAB-LRMS (glycerol matrix) *m/z* 554 (M + H); FAB-HRMS *m/z* 554.2864 (obsd), 554.2866 (calcd for $C_{30}H_{40}N_3O_7$); ^1H NMR (600.05 MHz in DMSO-d_6) δ 7.60 (2H, m, N-H, N′-H), 7.52 (1H, m, N″-H), 7.09 (2H, m, C_ϵ′-H, C_η′-H), 7.02 (3H, m, C_δ′-H, C_ξ′-H, C_θ′-H), 6.96 (1H, m, C_δ-H), 6.77 (1H, m, C_θ-H), 6.70 (1H, m, C_η-H), 6.65 (1H, m, C_ϵ-H), 4.56 (2H, m, C_α-H, C_β″-H), 4.42 (1H, m, C_α″-H), 4.24 (1H, m, C_α′-H), 3.85 (1H, dd, *J* = 9.8, 2.9 Hz, C_2-H), 3.20 and 2.42 (2H, m, C_β-H_2), 2.51 (1H, m, C_β′-H_{2a}), 2.49 (1H, m, C_β′-H_{2b}), 1.98 (1H, m, C_γ″-H), 1.77 (1H, m, C_4-H), 1.35 and 1.25 (1H each, m, C_3-H_2), 0.97 (3H, d, *J* = 6.8 Hz, C_δ″-H_3′), 0.91 (3H, d, *J* = 6.3 Hz, C_ϵ″-H_3″), 0.89 (3H, d, *J* = 6.8 Hz, C_5-H_3), 0.87 (3H, d, *J* = 6.8 Hz, C_6-H_3); ^{13}C NMR (100.4 MHz in DMSO-d_6) δ 173.0 (very weak signal, C═O), 172.9 (s, C_1═O), 169.2 (s, C″═O), 168.7 (s, C′═O), 155.5 (s, C_ξ), 137.4 (s, C_γ′), 131.5 (d, C_θ), 130.8 (s, C_γ), 129.7 (d, C_δ), 129.0 (d, overlapped C_ϵ′, C_ξ′), 127.7 (d, overlapped C_η′, C_θ′), 125.8 (d, C_δ′), 118.9 (d, C_ϵ), 113.7 (d, C_η), 79.7 (d, C_β″), 69.3 (d, C_2), 54.5 (d, C_α), 53.9 (d, C_α″), 53.0 (d, C_α′), 43.3 (t, C_3), 38.6 (t, C_β′), 37.7 (t, C_β), 27.8 (d, C_γ″), 23.9 (d, C_4), 23.5 (q, C_5), 21.3 (q, C_6), 20.4 (q, C_δ″), 14.9 (q, Cϵ″).

Vignatic Acid B (2): white powder; t_R 11.0 min; $[\alpha]_D$ −79° (*c* 0.16, MeOH); FAB-HRMS (glycerol matrix) *m/z* 520 (M + H); FAB-HRMS *m/z* 520.3010 (obsd), 520.3023 (calcd for $C_{27}H_{42}N_3O_7$); ^1H NMR (600.05 MHz in DMSO-d_6) δ 7.78 (1H, d, *J* = 10.2 Hz, N-H), 7.46 (1H, d, *J* = 9.8 Hz, N″-H), 7.25 (1H, d, *J* = 9.8 Hz, N′-H), 6.96 (1H, dd, *J* = 8.3, 2.4 Hz, C_δ-H), 6.89 (1H, dd, *J* = 8.8, 2.4 Hz, C_θ-H), 6.74 (1H, dd, *J* = 8.8, 2.4 Hz, C_η-H), 6.66 (1H, dd, *J* = 8.3, 2.4 Hz, C_ϵ-H), 5.58 (1H, d, *J* = 5.9 Hz, OH), 4.70 (1H, m, C_α-H), 4.62 (1H, dd, *J* = 9.3, 1.9 Hz, C_β″-H), 4.38 (1H, dd, *J* = 10.3, 9.8 Hz, C_α″-H), 4.04 (1H, m, C_α′-H), 3.88 (1H, m, C_2-H), 3.20 (1H, dd, *J* = 13.2, 5.9 Hz, C_β-H_{2a}), 2.43 (1H, m, C_β-H_{2b}), 2.01 (1H, m, C_γ″-H), 1.69 (1H, m, C_4-H), 1.30 (2H, m, C_3-H_2), 1.23 (1H, m, C_γ′-H), 1.15 (1H, m, C_β′-H_{2a}), 1.05 (1H, m, C_β′-H_{2b}), 1.01 (3H, d, *J* = 6.8 Hz, C_δ″-H_3′), 0.90 (3H, d, *J* = 6.8 Hz, C_ϵ″-H_3″), 0.83 (3H, d, *J* = 6.3 Hz, C_6-H_3), 0.81 (3H, d, *J* = 6.8 Hz, C_5-H_3), 0.74 (6H, d, *J* = 6.3 Hz, C_δ′-H_3, Cϵ′-H_3); ^{13}C NMR (100.4 MHz in DMSO-d_6) δ 172.9 (s, C_1), 172.6 (s, C), 169.7 (s, C′), 169.5 (s, C″), 155.6 (s, C_ξ), 131.6 (d, C_θ), 130.6 (s, C_γ), 129.4 (d, C_δ), 119.2 (d, C_ϵ), 113.7 (d, C_η), 79.5 (d, C_β″), 69.4 (d, C_2), 54.0 (d, C_α″), 52.9 (d, C_α), 50.3 (d, C_α′), 43.2 (t, C_3), 42.2 (t, C_β′), 36.7 (t, C_β), 27.8 (d, C_γ″), 24.3 (q, C_5), 23.8 (d, C_4), 23.7 (d, C_γ′), 22.9 (q, C_{5b}), 22.2 (q, C_δ′), 21.6 (q, C_6), 20.3 (q, C_δ″), 14.8 (q, C_ϵ″).

RESULTS AND DISCUSSION

Both vignatic acid A (**1**) and B (**2**) were isolated by HPLC (Figure 1) and dried to yield white powders with $[\alpha]_D$ −99° and −79°, respectively. L-Phenylalanine from **1** and L-leucine from **2** were detected by amino acid analyses.

The structural elucidation of **2** was completed prior to that of **1** because NMR spectra of **2** were much clearer than those of **1**. FAB mass spectral analysis of **2** gave a predominant molecular ion corresponding to M + H (*m/z* 520). FAB-HRMS gave a molecular ion at *m/z* 520.3010 from which a molecular formula of $C_{27}H_{41}N_3O_7$ was deduced. This formula was supported by the 100 MHz ^{13}C NMR spectrum which showed 27 carbon signals (Table 1).

The 600 MHz ^1H NMR spectrum of **2** in DMSO-d_6 showed D_2O exchangeable three doublet amide proton signals associated with four doublet of doublet signals of aromatic protons and five doublet methyl protons. The $^1H-^1H$ correlations were determined by using FG-DQFCOSY, and results are given in Figure 3. In this experiment, the presence of three amino acids was expected such as δ 7.78 (N-H), 4.70 (C_α-H), 3.20/2.43 (C_β-H_2); δ 7.25 (N′-H), 4.04 (C_α′-H), 1.15/1.05 (C_β′-H_2);

Table 1. NMR Assignments of Vignatic Acid A (1) and B (2)

position	vignatic acid A (**1**)				vignatic acid B (**2**)		
	^{13}C NMR[a]	^{1}H NMR[b]	HMBC[c]		^{13}C NMR[a]	^{1}H NMR[b]	HMBC[c]
				Tyrosine			
N-H		7.60				7.78	
C═O	173.0[d]				172.6		4.70, 3.20, 2.43
C_α-H	54.5	4.56	3.20/2.42		52.9	4.70	3.20, 2.43
C_β-H_2	37.7	3.20/2.42	6.96		36.7	3.20/2.43	6.96, 6.89, 4.70
C_γ	130.8		6.70, 6.65		130.6		6.74, 6.66
C_δ-H	129.7	6.96			129.4	6.96	6.89
C_ϵ-H	118.9	6.65	6.70		119.2	6.66	6.74
C_ζ	155.5		6.96, 6.70		155.6		6.96, 6.89, 6.74
			6.65, 4.56				6.66, 4.62
C_η-H	113.7	6.70	6.65		113.7	6.74	6.66
C_θ-H	131.5	6.77	6.96		131.6	6.89	6.96
		Phenylalanine				Leucine	
N′-H		7.60				7.25	
C′═O	168.7		2.51/2.49		169.7		7.78, 4.04, 1.15, 1.05
C_α′-H	53.0	4.24	2.51/2.49		50.3	4.04	
C_β′-H_2	38.6	2.51/2.49	7.02		42.2	1.05/1.15	4.04, 0.74
C_γ′	137.4		7.09	C_γ′-H	23.7	1.23	0.74
C_δ′-H	125.8	7.02	7.02	C_δ′-H_3	22.9	0.74	0.74
Cϵ′-H	129.0	7.09	7.09, 7.02	C_ϵ′-H_3	22.2	0.74	0.74
C_ζ′-H	129.0	7.02					
C_η′-H	127.7	7.09	7.09				
C_θ′-H	127.7	7.02					
				3-Hydroxyleucine			
N″-H		7.52				7.46	
C″═O	169.2		4.56, 4.42[d]		169.5		7.25, 4.62, 4.38
C_α″-H	53.9	4.42			54.0	4.38	
C_β″-H	79.7	4.56	0.97, 0.91		79.5	4.62	4.38, 1.01, 0.90
C_γ″-H	27.8	1.98	0.97, 0.91		27.8	2.01	1.01, 0.90
C_δ″-H_3	20.4	0.97	4.56, 1.98, 0.91		20.3	1.01	0.90
C_ϵ″-H_3	14.9	0.91	4.56, 1.98, 0.97		14.8	0.90	1.01
				2-Hydroxyisocaproic Acid			
C_1═O	172.9		3.85, 4.42[d]		172.9		7.46, 5.58, 4.38, 3.88, 1.30
C_2-H	69.3	3.85			69.4	3.88	5.58, 1.30
C_3-H_2	43.3	1.35/1.25	1.77, 0.89, 0.87		43.2	1.30	5.58, 1.69, 0.83, 0.81
C_4-H	23.9	1.77	3.85, 0.89, 0.87		23.8	1.69	3.88, 1.30
C_5-H_3	23.5	0.89	1.77, 0.87		24.3	0.81	1.69, 0.83
C_6-H_3	21.3	0.87	1.77, 0.84		21.6	0.83	0.81
OH		nd[e]				5.58	

[a] 100 MHz in DMSO-d_6. [b] 600 MHz in DMSO-d_6. [c] HMBC in DMSO-d_6. [d] Very weak signal. [e] Not determined.

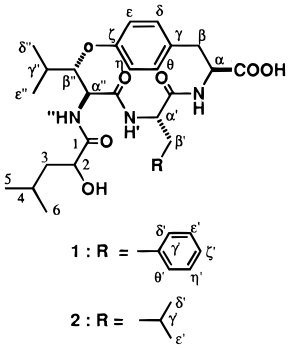

Figure 2. Structures of vignatic acid A (**1**) and B (**2**).

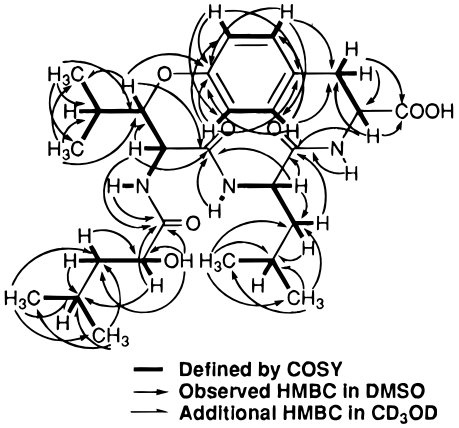

Figure 3. COSY and selected HMBC of **2** in DMSO-d_6 or CD_3-OD.

and δ 7.46 (N″-H), 4.38 (C_α″-H), 4.62 (C_β″-H), 2.01 (C_γ″-H), 1.01 (C_δ″-H_3)/0.90 (C_ϵ″-H_3), even though the connectivities between isopropyl proton signals and methylene proton signals of both leucine and 2-hydroxyisocaproic acid were missing.

The connectivities between ^{13}C and ^{1}H of **2** were determined by a PFG-HMQC experiment (Table 1). The gross structure of **2** was established by FG-HMBC (Table 1 and Figure 3). Two methyl proton signals of leucine at δ 0.74 (overlapped, C_δ′-H_3 and Cϵ′-H_3) showed cross peaks with a methine carbon at δ 23.7 (C_γ′) and a methylene carbon signal (C_β′) at δ 42.2, whereas two methyl proton signals of 2-hydroxyisocaproic acid at δ 0.83 (C_6) and 0.81 (C_5) had cross peaks with a methylene carbon signal (C_3) at δ 43.2, which was further connected with the hydroxy acid moiety having a carbonyl carbon signal (C_1) at δ 172.9.

The cross peaks between δ 4.70 (C_α-H) and 172.6 (COOH) of tyrosine, 4.04 (C_α′-H) and 169.7 (C′═O) of leucine, 4.38 (C_α″-H) and 169.5 (C″═O) of 3-hydroxyleucine, 3.88 (C_2-H) and 172.9 (C_1═O) of 2-hydroxyisocaproic acid, and 5.58 (O-H) and 172.9 (C_1) of 2-hydrox-

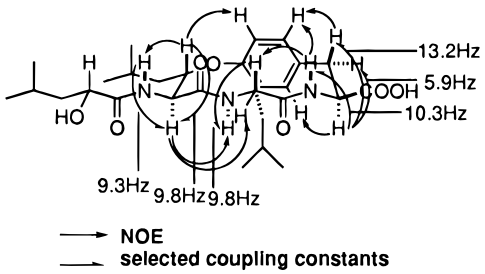

Figure 4. Observed NOE and selected coupling constants.

yisocaproic acid led to the assignments of four carbonyl carbon signals. The connectivities of three amino acids and an acid were established by the cross peaks between the amide proton signals and their neighboring carbonyl carbon signals such as δ 7.78 (N−H, tyrosine) and 169.7 (C′═O, leucine), 7.25 (N′-H, leucine) and 169.5 (C″═O, 3-hydroxyleucine), and 7.46 (N″-H) and 172.9 (C_1, 2-hydroxyisocaproic acid).

The lower-field-shifted methine proton signal (C_β″-H) at δ 4.62 had two cross peaks in DMSO-d_6 with a phenol carbon signal at 155.6 (C_ζ) of the tyrosine moiety and an amide carbonyl carbon signal at 169.5 (C″) of 3-hydroxyleucine. These correlations implied that 3-hydroxyleucine connected with the phenol moiety of tyrosine. Thus the whole structure of **2** was determined to be a cyclopeptide alkaloid containing tyrosine, leucine, 3-hydroxyleucine, and 2-hydroxyisocaproic acid.

The large coupling constants between δ 7.78 (N-H) and 4.70 (C_α-H), 7.25 (N′-H) and 4.04 (C_α′-H), 7.46 (N″-H) and 4.38 (C_α″-H), and 4.38 (C_α″-H) and 4.62 (C_β″-H) suggested that those protons are anti-parallel, whereas the observed NOEs between δ 7.78 (N-H) and 4.04 (C_α′-H), 7.25 (N′-H) and 4.38 (C_α″-H), and 7.46 (N″-H) and 4.62 (C_β″-H) implied that those protons are syn-parallel (Figure 4). Since **2** contains L-leucine, the other amino acids were determined to be L-tyrosine and 3-hydroxy-L-leucine. The secondary alcohol of 3-hydroxy-L-leucine was also determined to be *S* based on NOE-DF experiments starting from L-leucine, which is similar to some previously reported cyclopeptide alkaloids (Harborne and Baxter, 1993; Heffner et al., 1992). The absolute stereochemistry of 2-hydroxyisocaproic acid remains to be determined.

FAB mass spectral analysis of **1** yielded a predominant molecular ion corresponding to M + H (*m*/*z* 554). FAB-HRMS yielded a molecular ion at *m*/*z* 554.2864 from which a molecular formula of $C_{30}H_{39}N_3O_7$ was deduced. This formula was supported by the 100.4 MHz ^{13}C NMR spectrum which showed 30 carbon signals (Table 1). Analyses of the NMR spectra of vignatic acid A (**1**) were critical because the proton signals gave rather broad signals and some carbon signals were observed as overlapping peaks in DMSO-d_6.

After determination of C−H correlation by a PFG-HMQC experiment in DMSO-d_6 (Table 1), $^1H-^1H$ connectivities were established by FG-DQFCOSY, such as δ 7.60 (N-H), 4.56 (C_α-H), 3.20/2.42 (C_β-H_2); 7.60 (N′-H), 4.24 (C_α′-H), 2.55/2.49 (C_β′-H_2); 7.52 (N″-H), 4.42 (C_α″-H), 4.56 (C_β″-H), 1.98 (C_γ″-H), 0.97/0.91 (C_δ″-H_3′, C_ϵ″-H_3); and 3.85 (C_2-H), 1.35/1.25 (C_3-H_2), 1.77 (C_4-H), 0.87/0.89 (C_5-H_3, C_6-H_3). Two phenyl and two 2-butyl groups were assigned with the FG-HMBC in DMSO-d_6. The partial connectivities in the cyclopeptide moiety were observed, such as δ 2.51/2.49 (C_β′-H_2), 53.0 (C_α′), 168.7 (C′═O); 3.20/2.42 (C_β-H_2), 54.5 (C_α); 4.42 (C_α″-H), 169.2 (C″), 172.9 (C_1); 4.56 (C_β″-H), 169.2 (C″), 155.5 (C_ζ); and 3.85 (C_2-H), 172.9 (C_1). Although the connectivities concerning the amide protons were not observed in CD_3OD, FG-HMBC gave much better cross peaks, for example, from phenylalanine C_α′-H to both phenylalanine (C′) and hydroxyleucine (C_1) carbonyl carbon signals. It was suggested from the above analyses that the leucine in the structure of **2** was replaced by phenylalanine in that of **1**.

Table 2. Results of the Biological Assay by Using *C. chinensis*

concn of vignatic acid A (%)	adult emergence (%)	survival (%)
0	43.4	43.4
0.05	29.7	30.4
0.1	37.1	38.1
0.2	45.5	50.0
0.5	0	27.9
1	0	0

The absolute stereochemistry of **1** starting from L-phenylalanine was determined by the analyses of coupling constants and NOE-DF experiment. The large coupling constants a between δ 7.60 (N-H) and 4.56 (C_α-H), 7.60 (N-H) and 4.24 (C_α′-H), 7.52 (N-H) and 4.42 (C_α″-H), and 4.42 (C_α″-H) and 4.56 (C_β″-H) suggested that those protons are anti-parallel, whereas observed NOEs between δ 7.60 (N″-H) and 4.24 (C_α′-H), 7.60 (N′-H) and 4.42 (C_α″-H), and 7.52 (N″-H) and 4.56 (C_β″-H) implied that those protons are syn-parallel. Since **1** contains L-phenylalanine, the other amino acids were determined to be L-tyrosine and 3(S)-hydroxy-L-leucine. Although the absolute stereochemistry of 2-hydroxyisocaproic acid is not identified yet, those of L-tyrosine, L-phenylalanine, and 3(S)-L-hydroxyleucine were determined.

The extremely low yield of the FLEC derivative of 2-hydroxyisocaproic acid from either **1** or **2** was attributed to dehydration owing to the amino acid hydrolysis. A modified Mosher method (Trost et al., 1986) gave a complex mixture instead of the expected ester with *O*-methylmandelic acid, oxalyl chloride, and DMF in acetonitrile. Confirmation of the absolute structure of 2-hydroxyisocaproic acid is in progress by total synthesis of **1**.

The structures of **1** and **2** are classified as cyclopeptide alkaloids (Harborne and Baxter, 1993) possessing a 14-membered ring, e.g., frangulanine (Heffner et al., 1992). However neither has a free carboxylic acid on tyrosine and a hydroxyl acid on the side chain moiety.

The addition of the vignatic acid A (**1**) into artificial beans at a concentration of more than 1% resulted in the complete mortality of the bruchid, but vignatic acid B (**2**) was inactive with this content (Table 2). One question that remains unanswered is whether the insecticidal activity of vignatic acid A is enough to explain of the resistance of $B_{20}F_4$. Further investigation is needed to evaluate the principal factor in the resistance against azuki bean weevil. Neither **1** nor **2** showed cytotoxic effects on Chinese hamster ovarian tumor cell (CHO) and some human tumor cells (HT116 and K562) at 10 μg/mL. It should be noted that vignatic acid A (**1**) is the one and only cyclopeptide alkaloid isolated from plants to date which exhibits insect growth inhibition.

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LITERATURE CITED

Fujii, K.; Ishimoto, M.; Kitamura, K. *Appl. Entomol. Zool.* **1989**, *24*, 126−132.

Harborne, J. B.; Baxter, H. *Phytochemical Dictionary, A Handbook of Bioactive Compounds from Plants*; Taylor & Francis: London, 1993; Chapter 24.

Heffner, R. J.; Jiang, J.; Loullie, M. M. *J. Am. Chem. Soc.* **1992**, *114*, 10181−10189.

Ishimoto, M.; Kitamura, K. *Appl. Entomol. Zool.* **1989**, *24*, 281−286.

Ishimoto, M.; Kitamura, K. *Jpn. J. Breeding* **1993**, *43*, 75−80.

Kitamura, K.; Ishimoto, M.; Sawa, M. *Jpn. J. Breeding* **1988**, *38*, 459−464.

Trost, B. M.; Belletire, J. L.; Godleski, S.; McDougal, P. G.; Balkovec, J. M. *J. Org. Chem.* **1986**, *51*, 2370−2374.

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