Stephanotic Acid, a Novel Cyclic Pentapeptide from the Stem of *Stephanotis floribunda*

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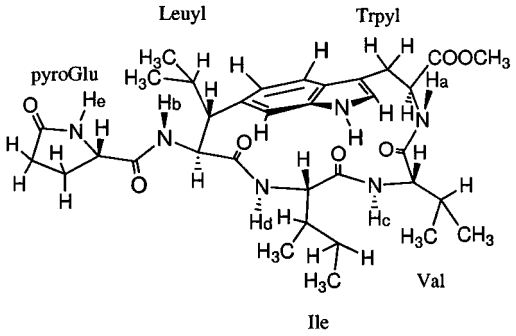
The stem of *Stephanotis floribunda* afforded a new cyclic pentapeptide stephanotic acid (**1**), possessing a novel 6-(leucin-3′-yl) tryptophan skeleton. The structure of **1** was assigned on the basis of extensive NMR experiments and a chemical reaction and shown to be closely related to the bicyclic octapeptide moroidin (**3**), a toxin from *Laportea moroides*.

The genus *Stephanotis* is represented by some 15 species in tropical areas (from the Malay Peninsula to Madagascar) with only one species reported in Japan. As part of our studies on natural antisweet substances occurring from Asclepiadaceous plants,[1] we have studied *S. floribunda* Brongn., which is used as an ornamental flower, corsage, and bouquet.[2] Though we could not get any antisweet substances from this plant, in the present paper we report the isolation and characterization of a new cyclic pentapeptide stephanotic acid (**1**), with a novel 6-(leucin-3′-yl)-tryptophan skeleton. Earlier phytochemical investigations of *Stephanotis* spp. were limited to antisweet substances[3−5] and steroidal glycosides.[6−11]

Stephanotic acid (**1**) showed a positive HRFABMS quasi-molecular ion peak at *m*/*z* 639.3507 {[M + H]$^+$, Δ + 0.2 mmu}, corresponding to the molecular formula $C_{33}H_{46}N_6O_7$. The IR spectrum contained typical peptide absorption bands at Δ 3300, 1665, and 1530 cm^{-1}. A negative ninhydrin test suggested that the *N*-terminus was blocked or part of a cyclic peptide, and a positive test against BTB indicates **1** to have a carboxy group. Its weak UV absorption at λ 292, 280, 275, and 231[12] and positive Ehrlich reaction[13] revealed the presence of an indole skeleton in **1**. Hydrolysis of **1** on 6 M HCl revealed pyroGlu, Ile, and Val by TLC analysis of the acid hydrolysate, while hydrolysis with 4 M CH_3SO_3H containing 0.2% 3-(2-aminoethyl)-indole[14] yielded L-Glu, L-Ile, and L-Val (1:1:1) by chiral HPLC analysis {CROWNPAK CR (+)} of the acid hydrolysate, indicating that **1** contained an unusual amino acid having an indole skelton. Methylation of **1** with diazomethane afforded compound **2**. Detailed analysis of the HOHAHA, HMBC, and NOESY spectra of **2** led to the complete assignment of all ^{1}H and ^{13}C NMR signals (Table 1). The unusual amino acid was determined to be a new amino, 6-(leucin-3′-yl) tryptophan (Leutrp) by the following experiments. The HOHAHA spectrum suggested these partial structures: **a** {NHCHCHCH(CH_3)CH_3 from C-2 to C-6 of leucin-3-yl (abbr. Leuyl) unit}: **b** {CH═CHC═CH from C-4 to C-7 of tryptophan-6-yl (abbr. Trpyl) unit}, and **c** (NHCH═CCH_2CHNH: from C-1 to C-α of Trpyl unit) (Figure 1). Connectivity between the **a** and **b** units was deduced from ^{1}H−^{13}C long-range couplings of H-3 (δ 3.01) of the Leuyl unit to C-5 (δ 118.9), C-6 (δ 129.7), and C-7 (δ 114.3) of the Trpyl unit in the HMBC spectrum of **2** (Figure 1). From these results, all five of the constituent amino acids in **2** were identified.



The peptide linkages of each unit were determined by HMBC and ROESY experiments. HMBC correlation was observed from *N*-Hb of Leuyl to CO of the pyroGlu−Leuyl residue. Another three HMBC correlation sets corresponded to *N*-Hd of Ile to CO of Leuyl, *N*-Hc of Val to CO of Ile, and *N*-Ha of Trpyl to CO of Val, allowing the cyclo-(-Val-Leutrp-Ile-) structure (Figure 1). Thus, the planar structure of **2** was determined. The relative stereochemistry of **2** was established by NOE observations between *N*-H and Hα of adjoining amino acids and the coupling constant values between *N*-H and Hα of each amino acid. The NOE between *N*-Hb of Leuyl and Hα of pyroGlu indicated the trans configuration of the peptide bond between Leuyl and pyroGlu. No significant NOE between Hα and Hβ of Leuyl and the coupling constant value between Hα and Hβ of Leuyl (^3J = 11.5 Hz) showed those protons to be trans. No significant NOE between *N*-Hc of Val and Hα of Ile suggested that they were trans coplanar. Strong NOEs between *N*-Hd of Ile and Hα of Leuyl and between N-Ha of Trpyl and Hα of Val showed that the peptide bonds between Leuyl and Ile and between Val and Trpyl were trans configurations, respectively (Figure 2). The coupling constants observed between *N*-Hd and Hα of Ile (^3J = 8.0 Hz), *N*-Hc and Hα of Val (^3J = 8.0 Hz) , and *N*-Ha and Hα of Trpyl (^3J = 8.0 Hz) indicated that in each core these protons were trans. As the structural details of **1** began to emerged, it was apparent that the new compound was related to moroidin (**3**), previously isolated from *Laportea moroides*.[15] The stereochemistry of moroidin (**3**) has been deduced by a combination of NMR and molecular modeling techniques by S. D. Kahn et al.[16] Comparison of the ^{1}H *J* values between *N*-H and Hα of amino acid and NOEs for **2** and **3** revealed that **2** possessed the same stereochemistry as **3**. Thus, the structure of stephanotic

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Table 1. ^{1}H and ^{13}C Spectral Data for Stephanotic Acid (**1**) and Methyl Stephanate (**2**)

		1 (pyridine-d_5)		**2** (DMSD-d_5)	
	position	^{1}H [int, mult, *J* (Hz)]	^{13}C(mult)	^{1}H [int, mult, *J* (Hz)]	^{13}C (mult)
Trpyl moiety	1	11.3 (1H, br s)		10.69 (1H, br s)	
	2	7.13 (1H, br s)	124.9 d	7.00 (1H, br s)	123.4 d
	3		110.5 s		108.3 s
	4	8.10 (1H, d, 8.5)	119.9 d	7.37 (1H, d, 8.0)	117.7 d
	5	7.50 (1H, br d, 8.5)	119.6 d	7.00 (1H, br d, 8.0)	118.9 d
	6		131.4 s		129.7 s
	7	7.47 (1H, br s)	115.8 d	6.89 (1H, br s)	114.3 d
	8		137.7 s		135.9 s
	9		127.7 s		125.4 s
	α	5.92 (1H, ddd, 8.5, 8.0, 5.0)	53.5 d	5.24 (1H, ddd, 8.5, 8.0, 5.5)	51.0 d
	β	3.66 (1H, dd, 15.0, 8.5)	28.6 t	3.14 (1H, dd, 15.0, 8.5)	28.9 t
	β	4.03 (1H, dd, 15.0, 5.0)		3.29 (1H, dd, 15.0, 5.5)	
	COO (H, CH_3)		176.2 s		171.1 s
	NHa	8.97 (1H, d, 8.0)		7.44 (1H, d, 8.0)	
	$COOCH_3$			3.66 (3H, s)	51.8 q
Leuyl moiety	1		172.7 s		169.8 s
	2	5.70 (1H, dd, 10.0, 9.0)	57.3 d	4.88 (1H, dd, 11.5, 9.0)	55.1 d
	3	3.60 (1H, dd, 10.0, 4.0)	53.2 d	3.01 (1H, dd, 11.5, 4.0)	51.7 d
	4	2.14 (1H, m)	28.6 d	2.14 (1H, m)	26.7 d
	5	0.79 (3H, d, 7.0)	21.7 q	0.79 (3H, d, 7.0)	21.7 q
	6	0.87 (3H, d, 6.5)	17.2 q	0.87 (3H, d, 6.5)	17.2 q
	NHb	9.65 (1H, d, 9.0)		8.53 (1H, d, 9.0)	
Val	1		171.2 s		170.5 s
	2	4.65 (1H, dd, 6.5, 6.0)	59.1 d	3.78 (1H, dd, 8.0, 6.0)	57.8 d
	3	2.57 (1H, m)	30.4 d	2.02 (1H, m)	30.4 d
	4	1.18 (3H, d, 6.5)	18.6 q	0.97 (1H, d, 6.5)	18.6 d
	5	1.19 (3H, d, 6.5)	17.8 q	0.94 (1H, d, 7.0)	17.8 d
	NHc	7.46 (1H, d, 6.0)		7.00 (1H, d, 8.0)	
Ile	1		171.9 s		169.6 s
	2	4.65 (1H, dd, 10.0, 6.0)	59.9 d	3.78 (1H, dd, 10.5, 8.0)	57.6 d
	3	2.09 (1H, m)	38.7 d	1.63 (1H, m)	38.7 d
	4	1.39 (1H, m)	25.6 t	0.95 (1H, m)	37.1 t
		1.73 (1H, m)		1.23 (1H, m)	
	5	0.79 (3H, d, 7.5)	11.4 q	0.68 (1H, d, 7.5)	10.3 q
	6	0.97 (3H, d, 7.0)	15.3 q	0.65 (1H, d, 7.0)	15.3 q
	NHd	9.21 (1H, d, 10.0)		8.31 (1H, d, 10.5)	
pyroGlu	1		174.2 s		172.1 s
	2	4.81 (1H, br dd, 8.0, 4.0)	57.3 d	4.12 (1H, br dd, 9.0, 4.0)	55.0 d
	3	1.73 (1H, m)	26.9 t	1.73 (1H, m)	25.5 t
		2.25 (1H, m)		2.25 (1H, m)	
	4	2.43 (1H, m)	30.4 t	2.08 (1H, m)	27.0 t
		2.66 (1H, m)		2.11 (1H, m)	
	5		180.0 s		177.3 s
	NHe	9.40 (1H, br s)		7.88 (1H, br s)	

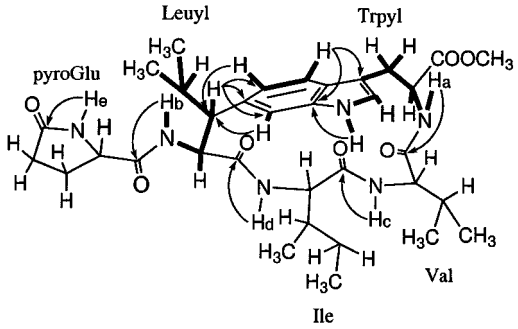

Figure 1. Key HOHAHA (bold lines) and HMBC (arrows) interaction of methyl stephanate (**2**).

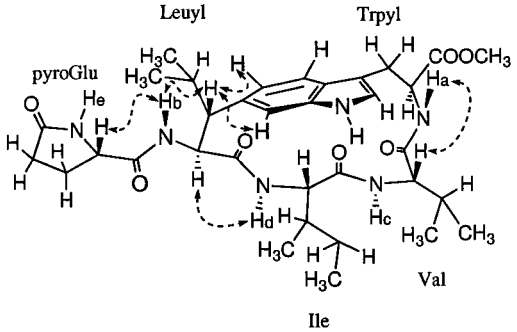

Figure 2. Key NOESY interactions of methyl stephanate (**2**).

acid was assigned as **1**. Compounds **1** and **2** were inactive (ED_{50} >50 mg/mL against KB and L1210 cells).

Experimental Section

General Experimental Procedures. The following instruments were used: a JASCO FT/IR-5300 (IR), a Shimadzu UV-160 (UV), a JASCO DIP-1000 polarimeter (optical rotation), a JASCO J-500C (CD), a JEOL JMS-HX-100 mass spectrometer (HRMS). ^{1}H and ^{13}C NMR, COSY, ROESY (mixing time 500 ms), HOHAHA (mixing time 80 ms), and HMBC ($^HJ_{CH}$-optimized for 8 Hz) spectra were recorded on a Varian UNITY 600 spectrometer.

Plant Material. *S. floribunda* was cultivated at the botanical garden of the Tokushima Bunri University, Tokushima. A specimen (TB 5424) is deposited at the Herbarium of the Department of Pharmacognosy, Tokushima Bunri University, Tokushima, Japan.

Extraction and Isolation. The fresh stems (4.5 kg) were extracted with 70% EtOH at the room temperature, then the solvent was evaporated under reduced pressure. The ethanolic extract (350 g) was dissolved in H_2O and fractionated by successive solvent extractions with hexane and then with

EtOAc. The H_2O-soluble material was subjected to a Daiaion HP-20 column (H_2O−MeOH). The 80% MeOH-eluted fraction was chromatographed on ODS−MPLC (50−80% MeOH) and finally by ODS−HPLC to isolate stephanotic acid (**1**, 500 mg, 0.11%).

Stephanotic acid (1): mp 250−252 °C; $[\alpha]^{25}_D$−143° (*c* 1.16, MeOH); UV (MeOH) λ 292 (3.72), 280 (3.79), 275 (3.76), 231 (4.31); CD (MeOH) $\Delta\epsilon$: +1.1 (287, peak), +0.63 (277, trough), +0.73 (270, peak), −8.5 (235, trough); FT−IR (dry film) 3300, 1665, 1530, 1235, 800, 700, 635 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; positive FABMS *m*/*z* 639 $[M + H]^+$, 661 $[M + Na]^+$; HRFABMS *m*/*z* $[M + H]^+$, 639.3507 (calcd for $C_{33}H_{46}N_6O_7$, 639.3509).

Methylation of 1. Compound **1** (20 mg) in MeOH (1 mL) was treated CH_2N_2 for 24 h at 4 °C. The residue, after solvent removal, was purified by preparative ODS−HPLC to give compound **2**: mp 202−204 °C; $[\alpha]^{25}_D$−118.6° (*c* 1.7, MeOH); UV (MeOH) λ 292 (3.42), 282 (3.50), 227.5 (3.76), 205 (4.18); FT−IR (dry film) 3300, 1740, 1665, 1520, 1265, 1215, 850, 710, 630 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HMBC (H/C) Trypyl moiety 1/2, 1/8, 1/9, 2/3, 2/8, 2/9, 2/β, 4/3, 4/6, 4/8, 4/9, 5/7, 5/9, 5/Leuyl 3, 7/5, 7/9, 7/ Leuyl 3, α/3, α/CO, β/2, β/3, β/9, β/α, β/CO, a/Val 1; Leuyl moiety 2/1, 2/3, 2/pyrGlu 1, 3/2−6, 3/Trypyl 5−7, 5/3, 5/4, 5/6, 6/3−5, b/2, b/3, b/pyrGlu 1; Val moiety 2/1, 2/3−5, 2/Ile 1, 3/1, 3/2, 3/4, 3/5, 4/2, 4/3, 4/5, 5/2−4, c/Ile 1: Ile moiety; 2/1, 2/3, 2/4, 2/6, 3/2, 3/4, 3/5, 4/2, 4/3, 4/5, 4/6, 5/2−4, 6/2, 6/3, 6/5, d/Leuyl 1; pyroGlu moiety 2/3, 2/5, 3/1, 3/4, 3/5, 4/2, 4/3, 4/5, e/2−4; positive FABMS *m*/*z* 653 $[M + H]^+$.

Acid Hydrolysis of 1 with 6 M HCl. A solution of **1** (2 mg) in 6 M HCl was heated at 150 °C for 18 h in a sealed tube. The residue after solvent removal showed three spots on TLC {*n*-BuOH−AcOH−H_2O (3:1:1)}, corresponding to isoleucine, pyroglutamic acid, and valine.

Acid Hydrolysis of 1 with 4 M Methanesulfonic Acid. A solution of **1** (2 mg) in 4 M CH_3SO_3H containing 0.2% 3-(2-aminoethyl) indole was heated at 115 °C for 22 h in a sealed tube. After hydrolysis, the hydrolysates were partially neutralized with 1.0 mL of 3.5 M NaOH and centrifuged for amino acid analysis. The mole ratio and D or L configuration of each amino acid was determined by using a chiral column {CROWNPAK CR (+), aqueous $HClO_4$, pH 2.0, 0.4 mL/min, 0 °C} by comparison with authentic amino acids (L-Glu, L-Ile, L-Val). These amino acids gave the following peaks: L-Val, 7.0 min; L-Ile, 13.0 min; L-Glu, 17.0 min.

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