Secondary Metabolites from Endophytic *Streptomyces* sp. Lz531

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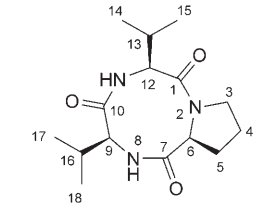
From the branch tissue of *Maytenus hookeri*, the endophytic strain Lz531 was isolated, and determined to belong to *Streptomyces*, according to its 16S rRNA sequence. From the extracts of the fermentation broth of *Streptomyces* sp. Lz531, two new and four known compounds were isolated. The two new compounds were identified as cyclo(L-Pro-L-Val-L-Val) (**1**) and 13-methyl-*N*-(2-phenylethyl)tetradecanamide (**2**).

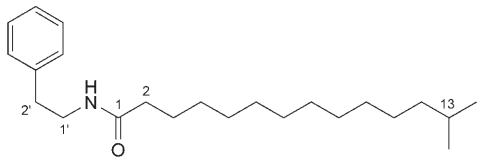
Introduction. – Endophytes, commonly present in almost all plants, are important sources of natural products [1]. *Maytenus hookeri* Loes. (Celastraceae), mostly distributed in China, India, and Burma, is well-recognized for producing the anticancer compound maytansine [2]. During our search for new bioactive natural products from the endophytic microorganisms of *M. hookeri*, a series of novel compounds were obtained [3–6]. In further studies concerning the chemical constituents of endophytic microorganisms from this plant, we investigated the secondary metabolites produced by the specific strain Lz531. Herein, we report the identification of *Streptomyces* sp. Lz531, and the isolation and structure elucidation of compounds **1**–**6**, including the new cyclic tripeptide **1** and the new amide **2**, which were obtained by chromatographic purification of the extracts from the corresponding fermentation broth.

Results and Discussion. – The Lz531 strain was identified by amplification of the corresponding 16S rRNA (see *Exper. Part*), and its 16S partial sequence was submitted to GenBank (accession No. EF125929). Blast search then showed that the sequence of Lz531 was highly homologous to other *Streptomyces* species, indicating that this strain is, indeed, a member of the genus *Streptomyces*.

Compound **1** was obtained as a colorless crystalloid. The HR-ESI-MS data indicated the molecular formula $C_{15}H_{25}N_3O_3$, based on the $[M+H]^+$ ion signal at *m*/*z* 296.1974 (calc. 296.1974). The NMR data (*Table 1*) revealed two quaternary C-atoms at δ(C) 170.1 and 164.9, and two N-substituted methines at δ(C) 58.8 and 60.4 (δ(H) 4.08 and 3.92, resp.), which suggested that compound **1** was a cyclic peptide. The NMR data of **1** were nearly identical to those of cyclo(D-Pro-D-Val) [7], but EI- and ESI-MS experiments did not show the expected M^+ or $[M+1]^+$ peaks at *m*/*z* 196 or 197, respectively. Based on the observed quasi-molecular signal at *m*/*z* 296, an MS/MS



1



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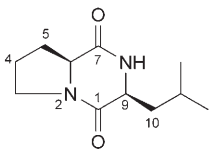

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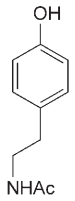

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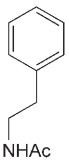

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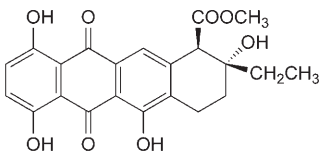

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experiment was performed (split mode), which gave rise to the loss of a fragment of *m*/*z* 86, affording a daughter ion at *m*/*z* 211, in accord with a Val–Pro fragment (*Fig.*). From these data, we anticipated that **1** was a cyclic tripeptide containing two Val and one Pro moieties.

Table 1. *NMR Data of* **1**. In $CDCl_3$; δ in ppm, *J* in Hz.

Position	δ(H)	δ(C)	HMBC
1		164.9	
3	3.55 (*dt*, *J* = 3.0, 9.0, H_β)	45.1	4
4	2.00–2.03 (*m*, H_α)	22.3	3, 6
	1.85–1.89 (*m*, H_β)		3, 5, 6
5	2.33–2.37 (*m*, H_α)	28.5	3, 4, 6
	2.00–2.03 (*m*, H_β)		4, 6
6	4.08 (*t*, *J* = 7.7)	58.8	4, 5, 7
7		170.1	
9	3.92 (*s*)	60.4	10, 16, 17, 18
10		164.9	
12	3.92 (*s*)	60.4	1, 13, 14, 15
13	2.60–2.63 (*m*)	28.4	1, 12, 14, 15
14	0.90 (*d*, *J* = 6.8)	16.0	12, 13, 15
15	1.07 (*d*, *J* = 7.2)	19.1	12, 13, 14
16	2.60–2.63 (*m*)	28.4	9, 10, 17, 18
17	0.90 (*d*, *J* = 6.8)	16.0	9, 16, 18
18	1.07 (*d*, *J* = 7.2)	19.1	9, 16, 17

The proposed structure of **1** was established by detailed HMQC and HMBC experiments (*Table 1*). The HMBC data[1]) showed correlations between δ(H) 4.08 (H–C(6)) and δ(C) 22.3 (C(4)), 28.5 (C(5)), and 170.1 (C(7)); and between δ(H) 3.92

[1]) Arbitrary atom numbering.

(H–C(9,12)) and δ(C) 16.0 (C(14,17)), 19.1 (C(15,18)), 28.4 (C(13,16)), and 164.9 (C(1,10)), among other correlations. The NOESY experiment showed interactions between H–C(6) and H–C(9,12), supporting the relative configurations at C(6) and C(9,12).

The absolute configuration of **1** was unambiguously determined to be (6*S*,9*S*,12*S*) by comparing the NMR data and specific optical rotation with the corresponding literature data of cyclo(L-Leu-L-Pro) and cyclo(D-Leu-D-Pro) [8], as well as cyclo(L-Ile-L-Pro-L-Leu-L-Pro) [9]. Based on the above data, the structure of **1** was elucidated as cyclo(L-Pro-L-Val-L-Val)[2]).

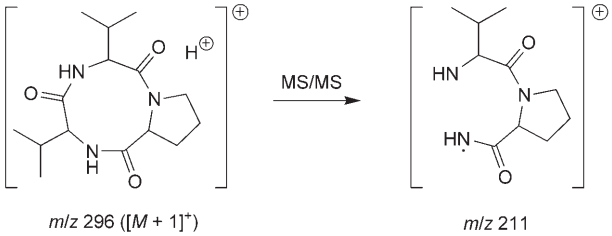

Figure. *MS/MS Fragmentation of* **1**

Compound **2** was obtained as a colorless powder. Its ^{1}H-NMR spectrum (*Table 2*) indicated five aromatic H-atoms [δ(H) 7.20 (*d*, *J* = 7.5, 2 H); 7.33 (*dd*, *J* = 7.8, 7.5, 2 H); 7.24 (*dd*, *J* = 7.8, 7.5, 1 H)], their coupling constants (*J* = 7.5–8.0 Hz) suggesting a mono-substituted phenyl ring. Further, an amide bond [δ(C) 173.1, 40.5; δ(H) 3.54] was present, as well as two Me groups [δ(H) 0.89 (*d*, *J* = 6.1, 6 H)]. By analysis of the

Table 2. *NMR Data of* **2**. In $CDCl_3$; δ in ppm, *J* in Hz.

Position	δ(H)	δ(C)	HMBC
1		173.1	
2	2.13 (*t*, *J* = 6.7)	36.9	1, 3
3	1.53–1.58 (*m*)	25.7	
4–11	1.20–1.25 (*m*)	29.9–29.4	
12	1.12–1.15 (*m*)	39.1	
13	1.20–1.25 (*m*)	27.4	
13-Me	0.89 (*d*, *J* = 6.1)	22.6	12, 13
14	0.89 (*d*, *J* = 6.1)	22.6	12, 13
1′	3.54 (*dt*, *J* = 6.3, 6.7)	40.5	1, 3′, 2′
2′	2.83 (*t*, *J* = 6.8)	35.8	3′, 4′/8′, 1′
3′		139.0	
4′	7.20 (*d*, *J* = 7.5)	128.6	5′, 6′, 2′
5′	7.33 (*dd*, *J* = 7.8, 7.5)	128.8	3′, 4′
6′	7.24 (*dd*, *J* = 7.8, 7.5)	126.5	5′/7′, 4′/8′
7′	7.33 (*dd*, *J* = 7.8, 7.5)	128.8	3′, 8′
8′	7.20 (*d*, *J* = 7.5)	128.6	7′, 6′

[2]) For the systematic name, see *Exper. Part.*

NMR data, compound **2** was shown to be the phenethylamide of a tetradecanoic acid [10].

In the HMBC spectrum of **2**, correlations between H–C(2′) and the C-atoms at δ(C) 139.0 (C(3′)), 128.6 (C(4′,8′)), and 40.5 (C(1′)) were observed, as well as between H–C(1′) and the signals at δ(C) 173.1 (C(1)), 139.0 (C(3′)), and 35.8 (C(2′)), confirming a phenethylamine moiety. In addition, the two Me groups correlated with the resonances at δ(C) 27.4 (C(13)) and 39.1 (C(12)). From these data, the structure of **2** was, thus, established as 13-methyl-*N*-(2-phenylethyl)tetradecanamide.

The structures of the known compounds **3**–**6** were determined as cyclo(L-Pro-L-Leu) (**3**) [8], *N*-acetyltyramine (**4**) [11], *N*-(2-phenylethyl)acetamide (**5**) [12], and 4-deoxy-ε-pyrromycinone (**6**) [13][14] by comparison with the corresponding literature data.

Experimental Part

General. TLC: Precoated *Si gel G* plates (*Qingdao Marine Chemical Factory*, Qingdao, China). Column chromatography (CC): *Sephadex LH-20* (*Pharmacia*); reverse-phase C_{18} (*RP-18*) silica gel (*Merck*); or silica gel *G* (200–300 mesh) and *H* (*Qingdao Marine Chemical Factory*). Optical rotation: *Jasco DIP*-370 polarimeter. NMR Spectra: *Bruker AM-400* and *DRX-500* spectrometers; δ in ppm rel. to Me_4Si, *J* in Hz. HR-ESI- and ESI-MS: *VG Auto-Spec-3000* and *Finnigan LCQ-Advantage* mass spectrometers, resp.; in *m*/*z*.

Microbial Material. The seeds of *Maytenus hookeri* were collected at Xishuangbanna (Yunnan Province, P. R. China) in April 2003. The material was washed under running tap water, then sterilized successively with 75% aq. EtOH (1 min) and 1.2% sodium hypochlorite (8 min), and then rinsed with sterile H_2O (5 ×). The sterilized plant material was cut into small pieces and incubated at 25° on *YMG* medium, prepared from yeast extract (4 g), malt extract (10 g), glucose (4 g), and agar (15 g) in dist. H_2O (1 l). The material was then cultured until colony or mycelium appeared, surrounding the segments. After culturing for *ca.* one month, a strain named Lz531 had appeared, and was isolated from the sterilized branch. A voucher sample was deposited at the Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, P. R. China.

Identification of the Lz531 Strain by Amplification of 16S rRNA. The mycelia of Lz531 were ground to a fine powder with liquid N_2. Then, the material was lysed for 2 h by exposure to 1% SDS in buffer *A* (500 mM *Tris*·HCl (pH 8.0), 20 mM EDTA, 10 mM NaCl) containing 200 μg/ml of proteinase K (*Merck*). The samples were extracted by phenol/$CHCl_3$ treatment, and precipitated with AcONa/EtOH by standard methods [15]. PCR was performed in a total volume of 50 μl, using the primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′). The reaction mixture containing DNA template (100 ng), primers (0.5 μM), dNTP (0.2 mM), 1 × *Ex-Taq* buffer (*Takara*), and *Ex-Taq* (1.25 U; *Takara*) was incubated in a thermal cycler (*Eppendorf*) as follows: 10 min of pre-denaturation; 25 cycles of 1 min of denaturation at 95°, annealing at 55° for 1 min, and elongation for 1.5 min; then 10 min of additional extension at 72°. The 1.5-kb PCR products were recovered by gel purification on a *UNIQ-10* column using a DNA gel-extraction kit (*Shanghai Sangon Biotechnology Co., Ltd*), and ligated into pUCm T vector (*Sangon*). Chemically competent *E. coli JM109* was prepared, and plasmids were introduced by a standard method [15]. Three randomly picked clones were sequenced on an *ABI-3700* sequencer for insert fragment. The 16S rDNA partial sequence was assembled using the Vector NTI software, and blasted against the latest GenBank database, using BLASTn.

Compound Extraction and Isolation. The Lz531 strain was cultured on *YMG* liquid medium (20 l) for 14 d. The cultures were filtered, and the filtrate was extracted successively with AcOEt (5 ×) and MeOH (5 ×) to afford, after solvent removal, 2.5 g and 5.6 g of AcOEt- and MeOH-soluble extracts. The AcOEt-soluble part (2.5 g) was subjected to CC (80 g SiO_2 *G*; 1. petroleum ether (PE)/acetone 10:1 →

7:1; 2. $CHCl_3$/MeOH 10:1→8:2) to afford eight fractions: *Fr. A1–A8*. *Fr. A3* (49 mg) was re-subjected to CC (8 g SiO_2 *G*; 1. PE/acetone 10:1→7:3; 2. 30 g *Sephadex LH-20*; acetone) to afford **2** (9 mg). *Fr. A4* was subjected to CC (SiO_2 and *Sephadex LH-20*) to provide **5** (4 mg). *Fr. A5* (68 mg) was purified by CC (1. 30 g *Sephadex LH-20*, MeOH; 2. 3 g SiO_2 *G*, PE/acetone 8:2→6:4) to provide **4** (7 mg).

The above MeOH-soluble part (5.6 g) was dissolved in MeOH and fractionated by MPLC (125 g *RP-18*; H_2O/MeOH gradient) to afford nine fractions: *Fr. M1–M9*. *Fr. M1* (860 mg) was subjected to CC (30 g *Sephadex LH-20*; MeOH) to afford eight fractions: *Fr. M1.1–M1.8*. *Fr. M1.2* (155 mg) was purified by CC (4 g SiO_2 *H*; PE/acetone 10:1→7:3) to afford three subfractions: *Fr. M1.2.1–M1.2.3*. From *Fr. M1.2.2* and *M1.2.3*, compounds **1** (8 mg) and **3** (5 mg), resp., were obtained by recrystallization. *Fr. M5* (176 mg) was subjected to CC (1. 50 g SiO_2 *G*, PE/acetone 9:1→7:3; 2. *Sephadex LH-20*, acetone) to provide **6** (56 mg).

*Cyclo(*L*-Pro-*L*-Val-*L*-Val)* (=*(3S,6S,11aS)-Octahydro-3,6-bis(1-methylethyl)-1H-pyrrolo[1,2-a][1,4,7]triazonine-1,4,7-trione*; **1**). Colorless crystalloid. $[\alpha]_D^{23} = +23.7$ ($c = 0.7$, $CHCl_3$). ^{1}H- and ^{13}C-NMR: see *Table 1*. ESI-MS: 318 ($[M+Na]^+$), 296 ($[M+H]^+$). HR-ESI-MS: 296.1974 ($[M+H]^+$, $C_{15}H_{26}N_3O_3^+$; calc. 296.1974).

*13-Methyl-*N*-(2-phenylethyl)tetradecanamide* (**2**). Colorless powder. $[\alpha]_D^{23} = +7.1$ ($c = 0.6$, $CHCl_3$). ^{1}H- and ^{13}C-NMR: see *Table 2*. ESI-MS: 346 ($[M+H]^+$, $C_{23}H_{40}NO^+$).

*Cyclo(*L*-Pro-*L*-Leu)* (**3**). Colorless crystalloid. $[\alpha]_D^{25} = -97.0$ ($c = 0.12$, $CHCl_3$). ^{1}H-NMR (400 MHz, CD_3OD): 0.92 (*d*, *J* = 5.8, Me(13)); 0.94 (*d*, *J* = 5.9, Me(12)); 1.90–1.95 (*m*, H_β–C(4)); 1.95–1.99 (*m*, H_β–C(5)); 2.03–2.05 (*m*, H_α–C(4)); 2.18–2.25 (*m*, H_α–C(5), H–C(11)); 2.86–2.90 (*m*, CH_2(10)); 3.55–3.60 (*m*, H_β–C(3)); 3.62–3.64 (*m*, H_α–C(3)); 4.05–4.08 (*m*, H–C(9)); 4.17 (*t*, *J* = 7.5, H–C(6)). ^{13}C-NMR (100 MHz, CD_3OD): 21.2 (C(12)); 22.4 (C(13)); 22.6 (C(4)); 24.4 (C(11)); 27.7 (C(5)); 38.3 (C(10)); 44.9 (C(3)); 53.1 (C(9)); 59.8 (C(6)); 166.3 (C(7)); 170.1 (C(1)). ESI-MS: 211 ($[M+H]^+$).

N-*Acetyltyramine* (**4**). Colorless powder. $[\alpha]_D^{23} = +5.0$ ($c = 0.6$, $CHCl_3$). ^{1}H-NMR (400 MHz, CD_3OD): 1.89 (*s*, Me); 2.69 (*t*, *J* = 7.5, CH_2(8)); 3.33 (*t*, *J* = 7.5, CH_2(7)); 7.02 (*d*, *J* = 9.2, H–(2,6)); 6.71 (*d*, *J* = 8.5, H–C(3,5)). ^{13}C-NMR (100 MHz, CD_3OD): 22.5 (Me); 35.7 (C(8)); 42.4 (C(7)); 116.2 (C(3,5)); 130.7 (C(2,6)); 131.2 (C(1)); 156.9 (C(4)); 173.2 (C=O). ESI-MS: 180 ($[M+H]^+$).

N-*(2-Phenylethyl)acetamide* (**5**). Colorless powder. $[\alpha]_D^{23} = +5.4$ ($c = 0.6$, $CHCl_3$). ^{1}H-NMR (400 MHz, CD_3OCD_3): 1.83 (*s*, Me); 2.78 (*t*, *J* = 7.6, CH_2(8)); 3.40 (*q*, *J* = 7.2, CH_2(7)); 7.25–7.28 (*m*, 5 arom. H). ^{13}C-NMR (100 MHz, CD_3OD): 22.9 (Me); 36.5 (C(8)); 41.5 (C(7)); 126.9 (C(4)); 129.5 (C(2,6)); 129.9 (C(3,5)); 140.6 (C(1)); 169.7 (C=O). ESI-MS: 164 ($[M+H]^+$).

4-Deoxy-ε-pyrromycinone (**6**). Red, amorphous powder. $[\alpha]_D^{23} = +84.0$ ($c = 0.5$, MeOH/$CHCl_3$ 1:1). ^{1}H-NMR (500 MHz, CD_3OD/$CDCl_3$ 4:1): 1.10 (*t*, *J* = 7.4, Me(14)); 1.63 (*q*, *J* = 7.2, CH_2(13)); 1.72–3.03 (*m*, CH_2(7,8)); 3.75 (*s*, MeO); 3.95 (*s*, H–C(10)); 7.27 (*s*, H–C(2,3)); 7.59 (*s*, H–C(11)). ^{13}C-NMR (125 MHz, CD_3OD/$CDCl_3$, 4:1): 6.6 (C(16)); 19.7 (C(7)); 27.6 (C(8)); 32.1 (C(15)); 52.0 (C(14)); 55.6 (C(10)); 71.1 (C(9)); 119.8 (C(5a)); 120.3 (C(2,3)); 128.8 (C(4a)); 129.0 (C(12a)); 129.3 (C(11)); 130.0 (C(6a)); 130.0 (C(11a)); 142.3 (C(10a)); 156.9 (C(1)); 157.3 (C(4)); 160.4 (C(6)); 171.7 (C(13)); 185.9 (C(5)); 190.5 (C(12)). ESI-MS: 411 ($[M-H]^-$).

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