Three New Polyketide Metabolites from the Endophytic Fungal Strain *Cladosporium tenuissimum* LR463 of *Maytenus hookeri*

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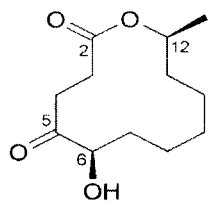
Three new polyketide metabolites, the twelve-membered macrolides (6*R*,12*S*)-6-hydroxy-12-methyloxacyclodoecane-2,5-dione (**1**), (10*S*,12*S*)-10-hydroxy-12-methyloxacyclododecane-2,5-dione (**2**), and 4,5-dihydroxy-12-methyloxacyclododecan-2-one (**3**), were isolated from the endophytic fungal strain *Cladosporium tenuissimum* LR463 of *Maytenus hookeri*, together with three known compounds, cladospolide A, cladospolide B, and isocladospolide B. Their structures were elucidated by spectroscopic analysis including 1D- and 2D-NMR experiments, and the absolute configurations of **1** and **2** were determined by the *Mosher* ester method.

1. Introduction. – Endophytic fungi were first found in pasture plants. Inoculation of watermelon and cucumber seedlings with non-pathogenic endophytic *Colletotrichum magna* rapidly induces systematic defense responses in plants, *e.g.*, the production of peroxidase, phenylalanine ammonia-lyase, lignin, and salicylic acid. Since endophytes constitute a valuable source of secondary metabolites for the discovery of new pharmaceuticals and lead compounds [1], we have started to investigate endophytic fungi as a source of biologically active natural products.

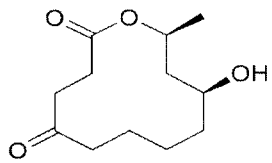
In this work, we report the isolation and structure elucidation of three new hexaketide lactones, **1**–**3**, which were isolated, together with three known compounds, **4**–**6**, from the fermented strain *Cladosporium tenuissimum* LR463 of *Maytenus hookeri*.

2. Results and Discussion. – The morphological properties of *C. tenuissimum* were examined after incubation at 28° for 10 d on potato-dextrose agar. This organism grew moderately on various agar media, and formed olive-gray to grayish-brown colonies of 50–60 mm in diameter. The colony surface was velvety to floccose, and the reverse of the colony was brownish gray. The fungal agar culture was extracted successively with petroleum ether, AcOEt, and BuOH. The AcOEt part was purified by column chromatography (*RP-18*, *Sephadex LH-20*, and silica gel) to afford three new compounds: cladospolide A (**1**), cladospolide B (**2**), and isocladospolide B (**3**).

Compound **1** was obtained as colorless needles (from $CHCl_3$). Its molecular formula was determined as $C_{12}H_{20}O_4$ (*m/z* 251.1259 ($[M+Na]^+$)) on the basis of HR-ESI-MS measurements, and confirmed by FAB-MS (*m/z* 229 ($[M+H]^+$)). The IR spectrum showed absorbances at 3455, 1700, and 1719 cm^{-1} due to OH, ester C=O, and keto C=O groups, respectively.



1



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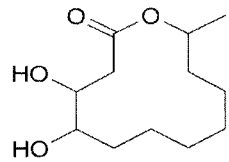
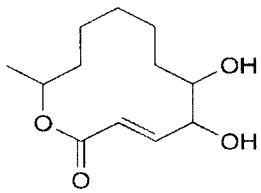
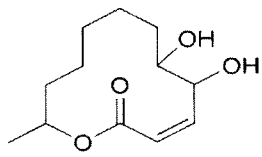


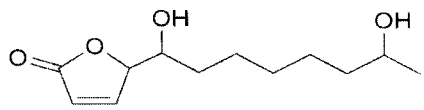
3



Cladospolide A (**4**)



Cladospolide B (**5**)



Isocladospolide B (**6**)

The ^{13}C-NMR (DEPT) spectrum of **1** showed twelve signals: one Me, seven CH_2, and two CH groups, and two quaternary C-atoms at δ(C) 171.5 (C(2)) and 213.6 (C(5)), indicating an ester and a keto functionality, resp. (*Table 1*). The ^{1}H-NMR spectrum contained two oxygenated-methine signals at δ(H) 4.06–4.09 (H–C(6)) and 4.74–4.76 (H–C(12)). ^{1}H,^{1}H-COSY and HMBC Experiments showed correlations between δ(H) 4.74–4.76 (H–C(12)) and 1.13 (12-Me), and between δ(H) 1.13 and δ(C) 72.2 (C(12)), resp., which suggested a Me group adjacent to an oxygenated methine (*Table 2*). Analysis of HMQC, ^{1}H,^{1}H-COSY, and HMBC experiments, and comparison of the spectroscopic data with those of cladospolides [2–4] [7] showed that compound **1** was a 12-membered macrolide. The absolute configuration at C(6) and C(12) was established by the *Mosher* test [5]. The ^{1}H-NMR signal of C(6) of the corresponding (*R*)-*Mosher* ester of **1** was shifted downfield ($\Delta\delta = -0.10$ ppm) relative to that of the (*S*)-configured ester, and the $\Delta\delta$ value for C(12) was 0.20. Thus, the (6*R*,12*S*)-configuration was assigned to **1**.

From the above data and by comparison with known cladospolides, the structure of **1** was determined as (6*R*,12*S*)-6-hydroxy-12-methyl-1-oxacyclododecane-2,5-dione (**1**).

Compound **2** was obtained as an oil. Its ESI mass spectrum showed the quasi-molecular ion peak at *m/z* 251 ($[M+Na]^+$), consistent with the molecular formula $C_{12}H_{20}O_4$, which was confirmed by ^{13}C-NMR (DEPT) experiments.

The IR spectra showed characteristic absorption bands for OH (3435), ester C=O (1715), and keto C=O (1669 cm^{-1}) groups. These assignments were further verified by NMR signals at δ(H) 3.78–3.82 (H–C(10)), δ(C) 171.4 (C(2)), and δ(C) 210.2 (C(5)). Comparison of the ^{1}H- and ^{13}C-NMR data of **2** with those of **1** suggested that the OH group was at C(10) in **2** (*Table 1*), as further corroborated by HMQC, ^{1}H,^{1}H-COSY, and HMBC experiments (*Table 2*). The absolute configuration at C(10) and C(12) was determined as for **1**, with $\Delta\delta(S) - \Delta\delta(R)$ values of the corresponding *Mosher* esters of +0.10 for C(10) and +0.30 for C(12), indicating (10*S*,12*S*)-configuration for **2**.

From the above data, HMQC, ^{1}H,^{1}H-COSY, and HMBC experiments, and by comparison with the data for **1**, the structure of **2** was identified as (10*S*,12*S*)-10-hydroxy-12-methyl-1-oxacyclododecane-2,5-dione.

Compound **3** was obtained as a colorless powder. The FAB mass spectrum showed the $[M+H]^+$ signal at *m/z* 231, in accord with the molecular formula $C_{12}H_{22}O_4$, as confirmed by HR-ESI-MS (*m/z* 253.1415 ($[M+Na]^+$)). The IR spectrum showed absorbances at 1723 (ester C=O) and 3385 (broad, OH) cm^{-1}. The ^{1}H- and ^{13}C-NMR spectra of **3** (*Table 1*) were similar to those of **1**, except for an additional oxygenated-methine

Table 1. *^{1}H- and ^{13}C-NMR Data for Compounds* **1**–**3**. At 400 and 100 MHz, resp., in (D_6)acetone; δ in ppm, J in Hz.

Position	**1**		**2**		**3**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
1	–	–	–	–	–	–
2	–	171.5 (*s*)	–	171.4 (*s*)	–	171.0 (*s*)
3	2.51–2.54 (*m*), 2.58–2.63 (*m*)	29.8 (*t*)	2.55–2.57 (*m*), 2.58–2.61 (*m*)	30.5 (*t*)	2.50–2.54 (*m*), 2.60–2.63 (*m*)	41.5 (*t*)
4	2.71–2.76 (*m*), 3.24–3.30 (*m*)	33.9 (*t*)	2.55–2.58 (*m*), 2.91–2.93 (*m*)	38.2 (*t*)	3.94–3.95 (*m*)	69.8 (*d*)
5	–	213.6 (*s*)	–	210.2 (*s*)	3.55–3.70 (*m*)	70.8 (*d*)
6	4.06–4.09 (*m*)	77.5 (*d*)	2.02–2.04 (*m*), 2.58–2.61 (*m*)	41.8 (*t*)	1.25–1.28 (*m*), 1.66–1.67 (*m*)	31.9 (*t*)
7	1.20–1.24 (*m*), 1.82–1.85 (*m*)	32.3 (*t*)	1.17–1.19 (*m*), 1.33–1.38 (*m*)	23.5 (*t*)	1.28–1.30 (*m*), 1.48–1.53 (*m*)	21.8 (*t*)
8	1.20–1.24 (*m*), 1.40–1.43 (*m*)	21.0 (*t*)	1.17–1.19 (*m*), 1.33–1.38 (*m*)	35.3 (*t*)	1.22–1.25 (*m*), 1.66–1.67 (*m*)	25.8 (*t*)
9	1.20–1.24 (*m*), 1.40–1.43 (*m*)	28.3 (*t*)	1.33–1.38 (*m*), 1.66–1.67 (*m*)	23.2 (*t*)	1.25–1.27 (*m*), 1.28–1.30 (*m*)	24.8 (*t*)
10	1.20–1.24 (*m*), 1.40–1.43 (*m*)	22.7 (*t*)	3.78–3.82 (*m*)	67.0 (*d*)	1.25–1.27 (*m*), 1.66–1.67 (*m*)	20.9 (*t*)
11	1.39–1.40 (*m*), 1.42–1.43 (*m*)	33.6 (*t*)	1.66–1.67 (*m*), 1.66–1.67 (*m*)	41.4 (*t*)	1.25–1.28 (*m*), 1.66–1.67 (*m*)	32.2 (*t*)
12	4.74–4.76 (*m*)	72.2 (*d*)	4.98–5.04 (*m*)	69.0 (*d*)	5.00–5.07 (*m*)	70.7 (*d*)
12-Me	1.13 (*d*, J=6.2)	20.1 (*q*)	1.19 (*d*, J=6.4)	20.4 (*q*)	1.13 (*d*, J=6.4)	19.0 (*q*)

group at δ(C) 69.8 (C(4)) and δ(H) 3.94–3.95 (H–(4)) in **3**, instead of a C=O resonance. Furthermore, 2D-NMR experiments confirmed the presence of OH groups in positions 4 and 5 (*Table 2*). The absolute configuration of **3** could not be derived yet due to the limited amount of material available. From these data, and by data comparison with **1** and **2**, the structure of **3** was deduced as 4,5-dihydroxy-12-methyl-1-oxacyclododecan-2-one.

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Experimental Part

General. Precoated TLC plates (*silica gel G*) were obtained from *Qingdao Marine Chemical Factory*, Qingdao, P. R. China. For column chromatography (CC), silica gel (200–300, and 80–100 mesh; *Qingdao Marine Chemical Factory*), silica gel *GF254* (*Merck*), *RP-18* (*Merck*), and *Sephadex LH-20* (*Amersham Biosciences*) were used. Melting points: *YuHua X-4* apparatus; uncorrected. UV Spectra: *Shimadzu double-beem 210A* spectral photometer; λ_{max} in nm. Optical rotations: *Jasco DIP-370* digital polarimeter. IR Spectra: *Bio-Rad FTS-135* IR spectrometer; with KBr pellets, in cm^{-1}. NMR Spectra: *Bruker AM-400* or *DRX-500* instruments; δ in ppm rel. to Me_4Si, J in Hz. MS: *VG Auto Spec-3000* spectrometer; in *m/z* (rel. %).

Table 2. *HMBC and $^1H,^1H$-COSY Correlations* for **1**–**3**

	1		**2**		**3**	
	HMBC	COSY	HMBC	COSY	HMBC	COSY
H_a–C(3)	C(2), C(5)	H–C(4)	C(4), C(5)	–	C(4), C(5)	H–C(4)
H_b–C(3)	–	–	C(4), C(2)	–	–	–
H_a–C(4)	C(3), C(5)	H–C(3)	C(2), C(3)	H–C(3)	C(3), C(6)	H–C(3), H–C(5)
H_b–C(4)	C(2)	–	C(5)	–	–	–
H–C(5)	–	–	–	–	C(3), C(6), C(7)	H–C(7)
H_a–C(6)	C(5), C(7)	H–C(7)	C(7), C(5)	H–C(7)	C(4), C(5)	H–C(7)
H_b–C(6)	–	–	C(4)	–	–	–
H_a–C(7)	C(5), C(8)	H–C(8)	C(6)	H–C(8)	–	–
H_b–C(7)	–	–	–	–	C(8), C(5)	–
H_a–C(8)	C(6), C(10)	H–C(7)	C(6), C(7)	H–C(9)	C(7), C(9)	H–C(7)
H_b–C(8)	C(6), C(10)	H–C(7)	C(10)	–	–	–
H_a–C(9)	C(8), C(10)	–	C(9), C(10)	H–C(10)	C(10), C(8)	–
H_b–C(9)	–	–	–	–	–	–
H_a–C(10)	C(12)		C(9), C(8)	H–C(9)	C(11), C(12)	H–C(12)
H_b–C(10)	–	–	–	–	–	H–C(11)
H_a–C(11)	C(10), C(12)	H–C(12)	C(12), C(10)	H–C(10), H–C(12)	C(9), C(10)	–
H_b–C(11)	–	–	–	–	–	H–C(10)
H–C(12)	C(2), C(11)	H–C(11)	C(2), C(10), C(11)	H–C(11), 12-Me	C(12), C(2)	12-Me
12-Me	C(11), C(12)	H–C(12)	C(11), C(12)	H–C(12)	C(11), C(12)	H–C(12)

Fermentation and Isolation of the Fungal Strain. The fungal strain LR463 of *C. tenuissimum* was isolated from the roots of *Maytenus hookeri* collected in Xishuangbanna Tropical Plant Garden, Chinese Academy of Sciences, Yunnan Province, P. R. China. Surface-sterilized samples were cut into 1-cm fragments, and ten fragments per sample, taken form different parts of the roots, were placed on 1.5% potato-dextrose-agar (PDA) medium. The plates were incubated at 28° for more than 7 d. The hyphal tips of the developing fungal colonies were transferred onto fresh PDA plates. After purifying the isolates several times, the final pure cultures were transferred to PDA slant tubes. The isolated fungus was identified as *C. tenuissimum* COOKE. The fungal strain was stored in 20% glycerol, and used to incubate on slope of PDA media in a test tube at 28° for 5 d to afford seed cultures. Solid-state fermentation was performed with *Sabouraud*'s agar medium (5 l; made from peptone (10 g/l), glucose (40 g/l), and agar (20 g/l)) at 28° for 14 d.

Extraction and Isolation. The cultured agar was chopped, diced, and extracted with AcOEt/MeOH/AcOH 80:15:5 (3×5 l) overnight. The org. solution was collected through filtration and concentrated *in vacuo* to afford 8.01 g (dry weight) of extract. The latter was taken up in H_2O (50 ml), and then extracted successively with petroleum ether, AcOEt, and BuOH (4×50 ml each). Each of the extracts was evaporated to dryness. The AcOEt extract (3.25 g) was subjected to CC (130 g *RP-18*), eluting with H_2O, and 30, 50, 70, and 100% MeOH, resp. (1.5 l each) to yield 5 fractions: *Fr. D1–D5*. *Fr. D3* (1.0 g) was subjected to CC (100 g *Sephadex LH-20*; MeOH). All fractions were analyzed by TLC ($CHCl_3$/MeOH 10:1), and pooled accordingly into three portions (*Fr. D3a–D3c*). *Fr. D3a* (25 mg) was further purified by VLC (2 g *GF254*); $CHCl_3$/acetone 100:3) and repeated CC (*Sephadex LH-20*; MeOH) to yield **1** (12 mg). *Fr. D3b* (100 mg) was subjected to CC (1. *RP-18*, MeOH/H_2O 30:70; 2. *Sephadex LH-20*, MeOH) to afford *Fr. D3b1* (30 mg) and *Fr. D3b2* (50 mg). *Fr. D3b1* was further purified by VLC (1. 1.5 g *GF254*, $CHCl_3$/MeOH 100:1; 2. *RP-18*, MeOH/H_2O 3:7) to yield **2** (6 mg). *Fr. D3b2* was further

purified by CC (*RP-18*; MeOH/H_2O 2:8) and VLC (2 g SiO_2, $CHCl_3$/MeOH 100:1) to yield **3** (4 mg) and cladospolide A (**4**; 15 mg). Cladospolide B (**5**; 8 mg) and isocladospolide B (**6**; 4 mg) were obtained from *Fr. D3c* through CC (*RP-18*; MeOH/H_2O 4:6).

Preparation of Mosher *Esters.* To a soln. of **1** (9 mg) or **2** (7 mg) in pyridine (0.2 ml) was added either (*R*)- or (*S*)-*Mosher* chloride[1]) (7 µl). The mixture was stirred at 25° for 24 h, evaporated, and purified by CC (*Sephadex LH-20*; acetone).

*(6*R*,12*S*)-6-Hydroxy-12-methyl-1-oxacyclododecane-2,5-dione* (**1**). Colorless needles ($CHCl_3$). M.p. 122–123°. UV (MeOH): 203. $[\alpha]_D^{19} = -39.3$ ($c = 0.57$, MeOH). IR (KBr): 3455, 2917, 1719, 1700, 1468, 1258. ^{1}H- and ^{13}C-NMR: see *Table 1*. FAB-MS: 229 (100, $[M+H]^+$), 211 (20, $[M-H_2O+H]^+$). HR-ESI-MS: 251.1256 ($[M+Na]^+$, $C_{12}H_{20}NaO_4^+$; calc: 251.1259).

*(10*S*,12*S*)-10-Hydroxy-12-methyl-1-oxacyclododecane-2,5-dione* (**2**). Colorless oil. UV (MeOH): 206. $[\alpha]_D^{26} = -54.5$ ($c = 0.91$, MeOH). IR (KBr) : 3435, 2933, 1715, 1669, 1466, 1263. ^{1}H- and ^{13}C-NMR: see *Table 1*. FAB-MS: 211 (65, $[M-H_2O+H]^+$), 288 (25). ESI-TOF-MS: 251 (100, $[M+Na]^+$), 211 (20, $[M-H_2O+H]^+$). HR-ESI-MS: 251.1257 ($[M+Na]^+$, $C_{12}H_{20}NaO_4^+$; calc: 251.1259).

4,5-Dihydroxy-12-methyl-1-oxacyclododecane-2-one (**3**). Colorless powder. M.p. 98–100°. UV (MeOH): 207. $[\alpha]_D^{26} = -29.5$ ($c = 0.8$, MeOH). IR (KBr): 3385, 2938, 1723, 1630, 1465, 1251. ^{1}H- and ^{13}C-NMR: see *Table 1*. FAB-MS: 231 (100, $[M+H]^+$), 223 (70), 242 (40), 207 (35). HR-ESI-MS: 253.1413 ($[M+Na]^+$, $C_{12}H_{22}NaO_4^+$; calc. 253.1415).

Cladospolide A (**4**). Colorless powder. The ^{1}H- and ^{13}C-NMR data were in agreement with those published in [6]. FAB-MS: 229 (100, $[M+H]^+$), 211 (60, $[M-H_2O+H]^+$).

Cladospolide B (**5**). Colorless powder. The ^{1}H- and ^{13}C-NMR data were in agreement with those published in [6]. ESI-TOF-MS: 251 (100, $[M+Na]^+$), 211 (20, $[M-H_2O+H]^+$).

Isocladospolide B (**6**). Colorless powder. The ^{1}H- and ^{13}C-NMR data were in agreement with those published in [5]. ESI-TOF-MS: 251 (100, $[M+Na]^+$).

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[1]) Systematic name: 3,3,3-Trifluoro-2-methoxy-2-phenylpropanoyl chloride.