Two New 7-Dehydrobrefeldin A Acids from *Cylindrocarpon obtusisporum*, an Endophytic Fungus of *Trewia nudiflora*

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Two new 7-dehydrobrefeldin A acids, (2*E*,4*R**)-4-hydroxy-4-{(1*R**,2*S**)-4-oxo-2-[(1*E*)-6-oxohept-1-en-1-yl]cyclopentyl}but-2-enoic acid (**3**) and (2*E*,4*R**)-4-hydroxy-4-{(1*R**,2*S**)-2-[(1*E*,6*S**)-6-hydroxy-hept-1-en-1-yl]-4-oxocyclopentyl}but-2-enoic acid (**4**), were isolated from the endophytic fungal strain *Cylindrocarpon obtusisporum* (COOKE & HARKNESS) WOLLENW. of *Trewia nudiflora*, together with two known compounds, 7-dehydrobrefeldin A (**2**) and brefeldin A (**1**). Their structures were determined on the basis of extensive 1D- and 2D-NMR-spectral analysis.

Introduction. – Endophytes, microorganisms that reside in the tissues of living plants, are relatively unstudied as potential sources of novel natural products for exploitation in medicine, agriculture, and industry [1][2]. During our continuing chemical and biological investigation of endophyte-generated secondary metabolites from *Trewia nudiflora* [3], an endophytic fungal strain, which was isolated from the roots of *T. nudiflora* and identified as *Cylindrocarpon obtusisporum*, was selected for further study because its agar fermentation extract showed strong antifungal activity against *Penicillium avellaneum* UC-4376. The subsequent bioassay-guided fractionation of the AcOEt fraction of the *Cylindrocarpon obtusisporum* culture extract led to the isolation of brefeldin A (**1**) and its analogues. In this article, we report two new 7-dehydrobrefeldin A acids and two known compounds: brefeldin A (**1**; *Fig. 1*) and 7-dehydrobrefeldin A (**2**).

Brefeldin A (**1**) is a 13-membered macrolide antibiotic which was isolated from various fungal species such as *Penicillium decumbens* [4], *Penicillium brefeldianum* [5], *Penicillium cyaneum* [6], and *Ascochyta imperfecta* [7]. For human beings, **1** was selected as a drug precursor, since it exhibits a diversity of biological activities which include antibiotics, antiviral, cytostatic, antimitotic, and antitumor effects [8]. For plants and animals, **1** was found to be able to inhibit the protein secretion at an early step in the secretory pathway [9].

Results and Discussion. – The fungal strain was cultivated on PDA (Potato Dextrose Agar) plates for 20 d at 26°. An AcOEt/MeOH/HCO_2H 80:15:5 extract of the culture was partitioned between AcOEt and H_2O. The AcOEt extract was chromatographed over *RP-18* gel, *Sephadex LH-20*, and silica gel to yield four compounds, **1**–**4**.

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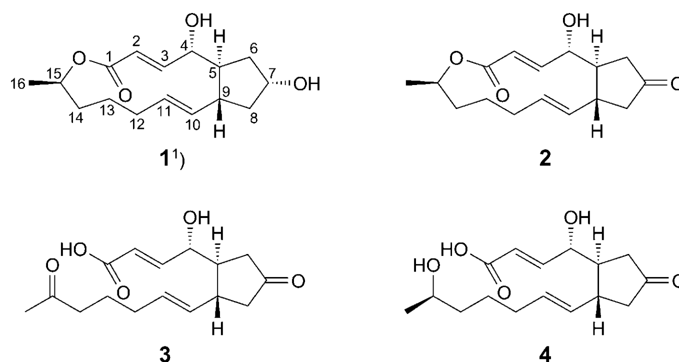

Fig. 1. *Structures of compounds* **1**–**4**

Compound **3**, obtained as colorless needles, had a molecular formula $C_{16}H_{22}O_5$ based on its HR-ESI-MS (*m*/*z* 317.1365 ([*M*+Na]$^+$)), indicating that **3** possesses six unsaturation degrees. The IR spectrum of **3** exhibited absorptions for COOH (3435 cm^{-1}), C=O (1711 cm^{-1}), and C=C (1658 cm^{-1}) groups. Inspection of the 1D- and 2D-NMR spectra (*Table*), and comparison with those of 7-dehydrobrefeldin A (**2**) [10] allowed us to identify the structure of compound **3**.

Analysis of the ^{13}C-NMR and DEPT spectra revealed that **3** contains 16 C-atoms, including two C=O (δ(C) 219.5, 211.9), one COOH (δ(C) 171.3), and two C=C groups (δ(C) 152.1, 133.3, 133.1, 121.5), one oxygenated CH (δ(C) 69.4), two non-oxygenated CH (δ(C) 48.5, 43.0), and five CH_2 groups (δ(C) 46.4, 43.5, 38.7, 32.8, 24.5), and one Me group (δ(C) 29.8). The NMR data were similar to those of the known compound **2**. The only difference between **2** and **3** was that the CH C-atom at C(15)[1]) of **2** was replaced by a CO group (δ(C) 211.9) in **3**.

According to the 1D-, 2D-NMR data and by comparison with the literature values, compound **2** was characterized as the known compound 7-dehydrobrefeldin A [10]. The relative configuration of **3** was determined by an NOE experiment and comparison with the NOE data of compound **2**. Key NOEs were observed between H–C(4) and H–C(9) and between H–C(5) and CH_2(6) (*Fig. 2*). H–C(9) does not have a correlation with H–C(5), but with H–C(4), which showed H–C(9)/H–C(5) may be on the different side of the molecule, and H–C(9)/H–C(4) on the same side. The similarity of NMR data between **3** and **2** also suggested that compound **3** might have the same configuration as compound **2**.

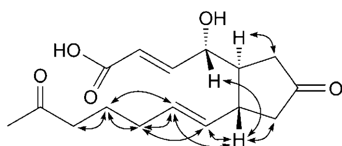

Fig. 2. *Key NOE correlations* (H↔H) *of compound* **3**

[1]) Arbitrary numbering. For systematic names, see *Exper. Part.*

Table. *^{1}H- and ^{13}C-NMR Data of Compounds* **3** *and* **4**[1]) (500 and 125 MHz, resp.; δ in ppm; J in Hz; in CD_3OD)

	3			**4**		
	δ(C)	δ(H)	HMBC (H → C)	δ(C)	δ(H)	HMBC (H → C)
1	171.3 (*s*)			169.9 (*s*)		
2	121.5 (*d*)	6.04 (*d*, J = 14.4)	C(1)	121.3 (*d*)	6.05 (*dd*, J = 1.0, 15.6)	C(1), C(3), C(4)
3	152.1 (*d*)	6.93 (*d*, J = 15.2)	C(1), C(2), C(4)	152.4 (*d*)	6.98 (*dd*, J = 4.2, 15.6)	C(1), C(2), C(4), C(5)
4	69.4 (*d*)	4.43 (*s*)	C(2), C(3), C(5), C(6), C(9)	69.3 (*d*)	4.47 (*d*, J = 1.2)	C(2), C(3), C(5), C(6), C(9)
5	48.5 (*d*)	2.12–2.19 (overlap)	C(8), C(10)	48.5 (*d*)	2.16–2.18 (overlap)	C(3), C(4), C(6), C(8)
6	38.7 (*t*)	2.12–2.17 (overlap)	C(4), C(5), C(7)	38.7 (*t*)	2.16–2.19 (overlap)	C(4), C(5), C(7), C(9)
		2.17–2.23 (overlap)	C(4), C(5), C(7)		2.22–2.29 (overlap)	C(4), C(5), C(7), C(9)
7	219.5 (*s*)			219.7 (*s*)		
8	46.4 (*t*)	2.44 (*dd*, J = 7.7, 18.3)	C(5), C(6), C(7), C(9), C(10)	46.5 (*t*)	2.47 (*dd*, J = 7.6, 18.3)	C(5), C(6), C(7), C(9), C(10)
		2.13–2.15 (overlap)	C(5), C(6), C(7), C(9), C(10)		2.16–2.20 (overlap)	C(5), C(6), C(7), C(9), C(10)
9	43.0 (*d*)	2.83–2.87 (*m*)	C(4), C(8), C(10), C(11)	43.1 (*d*)	2.86–2.92 (*m*)	C(4), C(5), C(8), C(11)
10	133.1 (*d*)	5.44 (*dd*, J = 8.2, 15.1)	C(5), C(8), C(9), C(11), C(12)	132.7 (*d*)	5.46 (*dd*, J = 8.4, 15.2)	C(5), C(8), C(9), C(11), C(12)
11	133.3 (*d*)	5.56–5.62 (*m*)	C(9), C(10), C(12), C(13)	134.0 (*d*)	5.62–5.68 (*m*)	C(9), C(10), C(12), C(13)
12	32.8 (*t*)	2.03–2.07 (*m*)	C(10), C(11), C(13), C(14)	33.6 (*t*)	2.06–2.11 (*m*)	C(11), C(13), C(14)
13	24.5 (*t*)	1.62–1.65 (*m*)	C(11), C(12), C(14), C(15)	26.7 (*t*)	1.48–1.54 (*m*)	CC(12), C(14), C(15)
14	43.5 (*t*)	2.50 (*t*, J = 7.3)	C(12), C(13), C(15)	39.6 (*t*)	1.41–1.54 (*m*)	C(12), C(13), C(15), C(16)
15	211.9 (*s*)			68.4 (*d*)	3.73–3.78 (*m*)	C(13), C(14)
16	29.8 (*q*)	2.12 (*s*)	C(14), C(15)	23.5 (*q*)	1.18 (*d*, J = 6.2)	C(14), C(15)

In the light of the evidence mentioned above and key HMBCs (*Fig. 3*), the structure of **3** was therefore established as shown in *Fig. 1* and the compound named (2*E*,4*R**)-4-hydroxy-4-{(1*R**,2*S**)-4-oxo-2-[(1*E*)-6-oxohept-1-en-1-yl]cyclopentyl}but-2-enoic acid.

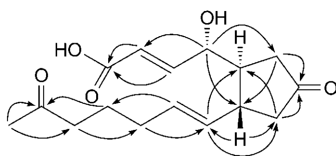

Fig. 3. *Key HMBCs of* (H → C) *compound* **3**

Compound **4**, obtained as colorless needles, had a molecular formula $C_{16}H_{24}O_5$ based on the HR-ESI-MS (*m*/*z* 319.1521 ([*M* + Na]$^+$)), indicating that **4** possesses five unsaturation degrees. The IR spectrum of **4** exhibited absorptions for COOH (3428 cm^{-1}), C=O (1731 and 1704 cm^{-1}), and C=C (1657 cm^{-1}) groups. Inspection of the 1D- and 2D-NMR spectra (*Table*) and comparison with those of compound **3** allowed us to identify the structure of compound **4**.

Analysis of the ^{13}C-NMR and DEPT spectra revealed that **4** contains 16 C-atoms, including one C=O (δ(C) 219.7), one COOH (169.9), and two C=C groups (δ(C) 152.4, 134.0, 132.7, 121.3), two oxygenated CH (δ(C) 69.3, 68.4), two non-oxygenated CH (δ(C) 48.5, 43.1), and five CH_2 groups (δ(C) 46.5, 39.6, 38.7, 33.6, 26.7), and one Me group (δ(C) 23.5). The NMR data were similar to those of compound **3**. The differences exist in that the C=O at C(15)[1]) (δ(C) 211.9) of **3** was replaced by a HO–CH group (δ(C) 68.4) in **4**. The relative configuration of **4** was determined by an NOE experiment as well as by the similarity with compound **2** and **3**. So, compound **4** was identified as (2*E*,4*R**)-4-hydroxy-4-{(1*R**,2*S**)-2-[(1*E*,6*S**)-6-hydroxyhept-1-en-1-yl]-4-oxocyclopentyl}but-2-enoic acid.

During the whole separation process, disk-diffusion assay on agar plates was used to track down the bioactivity compounds [11]. Anti-fungal bioassay-guided fractionation led to the isolation of compound **1**. At a concentration of 50 μg/disk, compound **1** showed antifungal activities against *Penicillium avellaneum* UC-4376 with inhibitory zones of 2.8 cm, whereas the positive control, nystain, showed inhibitory zones of 3.1 cm in diameter.

Experimental Part

General. TLC: precoated TLC plates (*Si gel G*) from *Qingdao Marine Chemical Factory*, Qingdao, P. R. China. Column chromatography (CC): silica gel (SiO_2; 200–300 and 80–100 mesh) from *Qingdao Marine Chemical Factory*, Qingdao, P. R. China; reversed-phase C_{18} silica gel from *Merck*; *Sephadex LH-20* from *Amersham Biosciences*. Optical rotations: *Jasco DIP-370* digital polarimeter. UV Spectra: *Shimadzu UV-2401PC* spectrophotometer. IR Spectra: *Bio-Rad FTS-135* IR spectrometer; KBr pellets; in cm^{-1}. NMR Spectra: *Brucker DRX-500* instrument; Me_4Si as internal standard; δ in ppm, *J* in Hz. MS: *VG Auto spec-3000-spectrometer*; in *m*/*z* (rel. %).

Biological Materials. The fungal strain was isolated from the roots of *Trewia nudiflora* L. (Euphorbiaceae), which were collected from a greenhouse of the Kunming Institute of Botany, P. R. China. The roots were washed in running tap water and cut into 5 mm × 5 mm pieces. These small pieces were surface-sterilized successively with 0.01% *Tween-20* for 30 s, 1% NaOCl soln. for 5 min, sterilized

H_2O for 5 min, and EtOH/H_2O (3 : 1) for 5 min. The surface-sterilized pieces were incubated at 26° on YMG (Yeast-Malt-Glucose) and cultivated until either a colony or mycelium appeared surrounding the pieces. 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 fungus was identified as *Cylindrocarpon obtusisporum* (COOKE & HARKNESS) WOLLENW by Prof. *Yunlong Liu*, Yunnan Agriculture University, Kunming, P. R. China, and deposited with the Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, P. R. China.

Culture Conditions and Extraction. Agar fermentation was performed with PDA medium (9 l) for 20 d. The cultured agar was chopped, diced, and extracted four times with AcOEt/MeOH/HCO_2H (80 : 15 : 5) exhaustively. The combined extracts were evaporated *in vacuo* at 45° to give a crude extract which was suspended in H_2O (0.5 l) and then extracted successively with petroleum ether (PE; 0.5 l × 3) and AcOEt (0.5 l × 3).

Isolation of the Compounds. The AcOEt extract (2.2 g) was subjected to CC (*C_{18}*; MeOH/H_2O 3 : 7, 5 : 5, 7 : 3, and 1 : 0) to give *Fractions 1–5*. *Fr. 2* (187 mg) was subjected to CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1 : 1) and then submitted to CC (SiO_2; PE/acetone 8 : 1 and 3 : 1) to yield 4 fractions: *Frs. 2a–2d*. *Fr. 2b* (57 mg) was subjected to CC (*Sephadex LH-20*; MeOH) and then purified by CC (SiO_2; $CHCl_3$/MeOH 1 : 0 and 9 : 1): **2** (10 mg). *Fr. 2c* (18 mg) was subjected to CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1 : 1) and then purified by VLC (*C_{18}*; MeOH/H_2O 7 : 13): **3** (7 mg). *Fr. 2d* (72 mg) was repeatedly purified by CC (*Sephadex LH-20*; MeOH, then *Sephadex LH-20*; acetone): **4** (19 mg). *Fr. 3* (210 mg) was fractionated by CC (SiO_2; $CHCl_3$/MeOH 50 : 1 and 20 : 1): *Frs. 3a–3c*. *Fr. 3c* was further purified by VLC (SiO_2; $CHCl_3$/MeOH 100 : 1 and 80 : 1): **1** (28 mg).

(2E,4R)-4-Hydroxy-4-{(1R*,2S*)-4-oxo-2-[(1E)-6-oxohept-1-en-1-yl]cyclopentyl}but-2-enoic Acid* (**3**). Colorless needles. $[\alpha]_D^{25} = -65.2$ ($c = 1.58$, MeOH). UV (MeOH): 204.8 (3.32). IR (KBr): 3435, 2932, 1711, 1658, 1403, 1370, 978. ^{1}H-NMR (500 MHz, MeOD): *Table*. ^{13}C-NMR (125 MHz, CD_3OD): *Table*. HR-ESI-MS: 317.1365 ($C_{16}H_{22}NaO_5^+$; calc. 317.1364).

(2E,4R)-4-Hydroxy-4-{(1R*,2S*)-2-[(1E,6S*)-6-hydroxyhept-1-en-1-yl]-4-oxocyclopentyl}but-2-enoic Acid* (**4**). Colorless needles. $[\alpha]_D^{25} = -52.1$ ($c = 0.21$, MeOH). UV (MeOH): 204.0 (4.06). IR (KBr): 3428, 2967, 2931, 1731, 1704, 1657, 980. ^{1}H-NMR (500 MHz, CD_3OD): *Table*. ^{13}C-NMR (125 MHz, MeOD): *Table*. HR-ESI-MS: 319.1521 ($C_{16}H_{24}NaO_5^+$; calc. 319.1521).

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