New Polyketides Isolated from *Botryosphaeria australis* Strain ZJ12-1A

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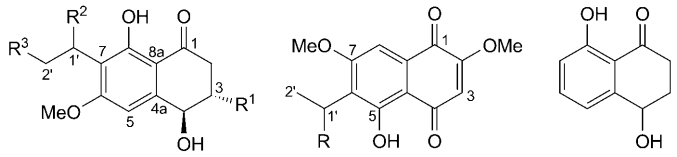
Four new polyketides, botryosphaerones A–D (**1**–**4**, resp.), were obtained from the fermentation culture of *Botryosphaeria australis* strain ZJ12-1A, together with four known compounds, *O*-methylasparvenone (**5**), 6-ethyl-2,7-dimethoxyjuglon (**6**) and its monoacetyl derivative **7**, and *O*-methylaspmenone (**8**). Their structures were elucidated by spectroscopic analyses, including 1D- and 2D-NMR experiments, and HR-Q-TOF mass spectrometry, and by comparison with reported data. All compounds were evaluated for their cytotoxic and antimicrobial activities *in vitro*. Only compounds **6** and **7** showed cytotoxic and antimicrobial activities, as already reported.

Introduction. – Plant-associated fungi, including endophytic and epiphytic fungi, are important sources of bioactive compounds. Previously, we mainly focused on the bioactive metabolites from endophytic fungi [1–5]. In our search for antitumor compounds from epiphytic fungi, we observed that the fermentation extracts of strain ZJ12-1 exhibited strong cytotoxic activity. This strain ZJ12-1A was originally obtained from the plant epidermis of *Sonneratia apetala* BUCH. HAM (a kind of mangroves), and it was identified as *Botryosphaeria* sp. based on its complete ITS4-5.8S-ITS5 gene sequences. Various compounds were isolated from the genus *Botryosphaeria*. Some of them showed interesting biological activities; *i.e.*, antifungal and cytotoxic depsidones [6], antimicrobe diterpenoids [7] and antiseptic mellein [8], antimicrobial lasiodiplodin [9], dihydronaphthalenones [10], and dihydrobenzofuran, and antibacterial primin [11]. The current study on the chemical constituents of strain ZJ12-1A resulted in the isolation of four new naphthalenones, named botryosphaerones A–D (**1**–**4**, resp.), together with four known compounds **5**–**8** (*Fig. 1*). Here, we report the isolation and structure elucidation of compounds **1**–**4**. The *in vitro* antimicrobial and cytotoxic testing of compounds **1**–**8** are also described.

Results and Discussion. – 1. *Structure Elucidation*. *Botryosphaeria australis* strain ZJ12-1A was cultivated for 14 d in *Petri* dishes with PDA (potato dextrose agar) medium and a total of 10 l at 28°. The fermentation culture was extracted with AcOEt/MeOH/AcOH 80 : 15 : 5 (*v*/*v*/*v*). The crude extract was partitioned between AcOEt and H_2O. The AcOEt-soluble fraction was dried over Na_2SO_4 (anh.) and concentrated *in vacuo* to afford 12.6 g of a crude org. extract (brown solid). The extract was purified by



	R^1	R^2	R^3
1	OH	OH	H
2	OH	H	OH
3	OH	MeO	H
4	OH	H	H
5	H	H	H

6 R = H
7 R = AcO

8

Fig. 1. *The structures of compounds* **1**–**8**

repeated column chromatography (*RP-18*, *Sephadex LH-20*, and SiO_2) to afford four new and four known polyketides.

Compound **1** was obtained as a white solid. The molecular formula of **1** was determined as $C_{13}H_{16}O_6$ on the basis of HR-Q-TOF-MS and NMR data. The IR absorption at 3369 cm^{-1} indicated the presence of OH groups. The ^{13}C-NMR (DEPT) spectrum of **1** (*Table 1*) exhibited 13 signals corresponding to two Me, one CH_2, four CH (three of them O-bearing), groups and six quaternary C-atoms (three of them O-bearing, including one C=O group at δ(C) 202.3 (C(1)). Analysis of the ^{1}H-NMR spectrum of **1** (*Table 2*) indicated the presence of one Me (δ(H) 1.83) and one MeO group (δ(H) 3.74). The connectivity of the H- and C-atoms was established by a HSQC spectrum. HMBCs from Me(2′) to C(1′) and C(7), from H–C(1′) to C(6), C(7), and C(8), and from MeO–C(6) to C(6), together with the analysis of ^{1}H,^{1}H-COSY cross-peaks, afforded fragment **1a** (*Fig. 2*). Three OH groups were assigned to C(3) (δ(C) 71.5), C(4) (δ(C) 73.8), and C(1′) (δ(C) 62.3), according to the resonance of those C-

Table 1. *^{13}C-NMR Data of* **1**–**4**. Recorded at 150 MHz in C_5D_5N (for **1**, **2**, and **3**) or in (D_6)acetone (for **4**); δ in ppm, *J* in Hz.

	1	**2**	**3**	**4**
C(1)	202.3 (*s*)	202.2 (*s*)	202.5 (*s*)	201.2 (*s*)
C(2)	45.2 (*t*)	45.3 (*t*)	45.4 (*t*)	43.7 (*t*)
C(3)	71.5 (*d*)	71.7 (*d*)	71.7 (*d*)	70.8 (*d*)
C(4)	73.8 (*d*)	74.1 (*d*)	74.1 (*d*)	72.9 (*d*)
C(4a)	147.9 (*s*)	147.2 (*s*)	148.9 (*s*)	145.1 (*s*)
C(5)	102.2 (*d*)	102.0 (*d*)	102.5 (*d*)	101.2 (*d*)
C(6)	163.8 (*s*)	164.8 (*s*)	165.5 (*s*)	163.7 (*s*)
*Me*O–C(6)	55.6 (*q*)	55.7 (*q*)	55.9 (*q*)	55.3 (*q*)
C(7)	119.1 (*s*)	113.0 (*s*)	115.2 (*s*)	117.2 (*s*)
C(8)	161.7 (*s*)	162.6 (*s*)	163.2 (*s*)	161.2 (*s*)
C(8a)	111.1 (*s*)	111.2 (*s*)	111.2 (*s*)	110.1 (*s*)
C(1′)	62.3 (*d*)	27.4 (*t*)	70.6 (*d*)	15.1 (*t*)
*Me*O–C(1′)			56.2 (*q*)	
C(2′)	22.9 (*q*)	61.0 (*t*)	19.7 (*q*)	12.6 (*q*)

Table 2. *^{1}H-NMR Data of* **1**–**4**. Recorded at 600 MHz in C_5D_5N (for **1**, **2**, and **3**) or in (D_6)acetone (for **4**); δ in ppm, J in Hz.

	1	**2**	**3**	**4**
CH_2(2)	3.45 (*dt, J* = 3.3, 17.0), 3.11 (*dd*, *J* = 10.0, 17.0)	3.45 (*dd*, *J* = 4.3, 17.0), 3.13 (*dd*, *J* = 9.9, 17.0)	3.49 (*dt*, *J* = 4.5, 17.0), 3.14 (*dd*, *J* = 9.8, 17.0)	2.98 (*dd*, *J* = 4.4, 17.0), 2.70 (*dd*, *J* = 9.5, 17.0)
H–C(3)	4.48–4.53 (*m*)	4.52–4.56 (*m*)	4.52–4.57 (*m*)	4.01–4.06 (*m*)
H–C(4)	5.12 (*d*, *J* = 8.0)	5.15 (*d*, *J* = 7.9)	5.16 (*d*, *J* = 7.7)	4.61 (*d*, overlap)
H–C(5)	7.30 (*s*)	7.30 (*s*)	7.34 (*s*)	6.88 (*s*)
MeO–C(6)	3.74 (*s*)	3.74 (*s*)	3.78 (*s*)	3.94 (*s*)
H–C(1′)	5.86 (*dq*, *J* = 2.8, 6.7)	3.44 (*t*, *J* = 7.6)	5.37 (*dq*, *J* = 2.3, 6.7)	2.63 (*q*, *J* = 7.4)
MeO–C(1′)			3.33 (*s*)	
Me(2′) or CH_2(2′)	1.83 (*d*, *J* = 6.7)	4.23 (*t*, *J* = 7.6)	1.80 (*d*, *J* = 6.7)	1.06 (*t*, *J* = 7.4)

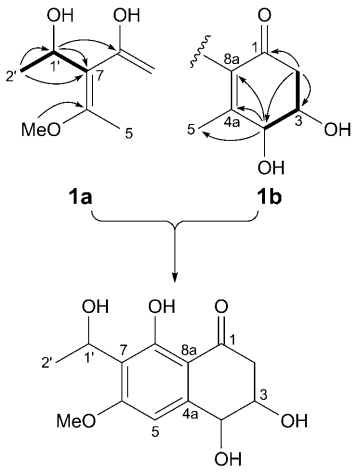


Fig. 2. *Structure fragments of* **1**, *and selected HMBC* (H → C) *and ^{1}H,^{1}H-COSY* (—) *correlations*

atoms at low field. The ^{1}H,^{1}H-COSY cross-peaks H–C(2)/H–C(3) and H–C(3)/H–C(4), and HMBCs from H–C(2) to C(1), C(3), and C(4), and from H–C(4) to C(4a), C(5), and C(8a) led to the fragment **1b** (*Fig. 2*). The HMBC experiments also showed ^{1}H,^{13}C long-range correlations from H–C(5) to C(4), C(4a), and C(8a) in fragment **1b**, and to C(6) and C(7) in fragment **1a**, indicating the connection of fragments **1a** and **1b** *via* C(5). The presence of an aromatic ring implied the linkage between C(8) and C(8a), providing the full planar structure of **1**.

The relative configuration of **1** was determined by the analysis of the NOESY spectrum. The NOESY correlation between H_a–C(2) and H–C(4) indicated that H_a–C(2) and H–C(4) were α-oriented, while the NOE correlation between H_b–C(2) and H–C(3) indicated β-orientations of these H-atoms (*Fig. 3*). However, the configuration at C(1′) remains undetermined. Thus, from the data above, the structure of compound **1** was determined as (3*S**,4*S**)-3,4-dihydro-3,4,8-trihydroxy-7-(1-hydroxyethyl)-6-methoxynaphthalen-1(2*H*)-one, named botryosphaerone A.

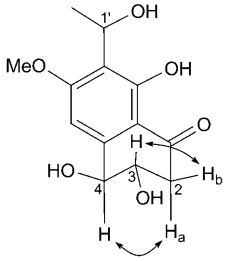


Fig. 3. *Selected NOESY correlations of compound* **1** (H ↔ H)

Compound **2** was obtained as a white solid. The molecular formula was determined as $C_{13}H_{16}O_6$ on the basis of HR-Q-TOF-MS and NMR data. The IR absorption at 3312 cm^{-1} indicated the presence of OH groups. The NMR data of **2** were similar to those of **1** (*Tables 1* and *2*), except that the resonances of C(1′) and C(2′) were shifted from δ(C) 62.3 to 27.4, and from δ(C) 22.9 to 61.0, respectively, revealing that the OH group at C(1′) in **1** was located at C(2′) in **2**. The relative configuration of **2** was determined on the basis of the same NOESY correlations as in **1**. Thus, compound **2** was determined to be (3*S**,4*S**)-3,4-dihydro-3,4,8-trihydroxy-6-methoxy-7-(2-hydroxyethyl)naphthalen-1(2*H*)-one, named botryosphaerone B.

Compound **3** was obtained as a white solid. The molecular formula was determined as $C_{14}H_{18}O_6$ on the basis of HR-Q-TOF-MS and NMR data. The IR absorption at 3384 cm^{-1} indicated the presence of OH groups. The NMR data of **3** were similar to those of **1** (*Tables 1* and *2*), except that the OH group at C(1′) in **1** was replaced by a MeO group in **3**, which was confirmed by the ^1H,^{13}C long-range correlations from the MeO H-atoms at δ(H) 3.33 to C(1′) at δ(C) 70.6. The configuration of **3** was determined based on the same NOESY correlations as in **1**. Thus, compound **3** was determined to be (3*S**,4*S**)-3,4-dihydro-3,4,8-trihydroxy-6-methoxy-7-(1-methoxyethyl)naphthalen-1(2*H*)-one, named botryosphaerone C.

Compound **4** was obtained as a white solid. The molecular formula was determined as $C_{13}H_{16}O_5$ on the basis of HR-Q-TOF-MS and NMR data. The IR absorption at 3428 cm^{-1} indicated the presence of OH groups. The NMR data of **4** (*Table 3*) were similar to those of **1** (*Tables 1* and *2*), except that the OH group at C(1′) in **1** was absent in **4**. The configuration of **4** was determined based on the same NOESY correlations as in **1**. Thus, compound **4** was determined as (3*S**,4*S**)-3,4-dihydro-3,4,8-trihydroxy-6-methoxynaphthalen-1(2*H*)-one, named botryosphaerone D.

Compounds **5**–**8** were identified as *O*-methylasparvenone (**5**) [12], 6-ethyl-2,7-dimethoxyjuglon (**6**) [13] and its monoacetyl derivative **7** [13], and *O*-methylaspmenone (**8**) [13–16] by comparison of the NMR data with those reported in literature [12–16].

2. *Biological Properties.* Compounds **1**–**8** were tested in cytotoxic and antimicrobial assays *in vitro*. The new compounds **1**–**4** exhibited no cytotoxic activities against HeLa, HepG-2, and A-549 cells at a concentration of 10 μg/ml, nor antimicrobial activities against pathogenic bacteria (*Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Bacillus pumilus*) or yeasts (*Candida albicans* and *Aspergillus niger*) at a concentration of 50 μg/ml. Compounds **6** and **7** showed cytotoxic and antimicrobial activities, as described in [17–19].

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Experimental Part

General. TLC: Precoated silica gel GF_{254} plates (0.2–0.25 mm, *Qingdao Marine Chemical Factory*, Qingdao, P. R. China). Column chromatography (CC): SiO_2 (200–300 and 80–100 mesh; *Qingdao Marine Chemical Factory*), SiO_2 GF_{254} (*Merck*), *RP-18* gel (40–63 μm; *Merck*), and *Sephadex LH-20* (*Amersham Biosciences*). Optical rotations: *Perkin-Elmer 341* polarimeter with MeOH as solvent. UV Spectra: *UniCO single-beam 210A* spectral photometer; 190–1100 nm, in MeOH. IR: in KBr on a *Nicolet Avatar 330*; in cm^{-1}. ^{1}H- and ^{13}C-NMR Spectra: *Bruker DRX 600* spectrometer, at 600 and 150 MHz, resp., in ppm rel. to Me_4Si, *J* in Hz. HR-Q-TOF-MS: *BioTOF*™-*Q* mass spectrometer (*Bruker*); in *m*/*z* (rel. %).

Isolation and Fermentation. *Botryosphaeria australis* strain ZJ12-1A was isolated from the plant epidermis of *Sonneratia apetala*, which was collected in the Xiamen Haicang mangrove Conservation Area, Fujian Province, P. R. China, in July, 2006. Surface-sterilized samples from the fresh roots were cut into 1-cm fragments, with ten fragments per sample. The sterilized samples were placed onto the surface of melted potato dextrose agar (PDA; 15 ml) as medium in *Petri* dishes, and then cultured at 28°. During cultivation, the hyphal tips of the growing fungi were removed, inoculated onto fresh PDA medium, and incubated for at least 1 week. After being purified by the hyphal-tip method. The pure isolates were transferred to PDA slant tubes as deposit. This organism was identified as a *Botryosphaeria* species, based on its complete ITS1-5.8S-ITS2 gene sequences (Genbank registered No. FJ037758.1). A stock of *Botryosphaeria australis* strain ZJ12-1A was cultured in dishes with *ca*. 20 ml of PDA medium with a total volume of 10 l for 14 d at 28°.

Extraction and Isolation. After fermentation on PDA plates for 14 d at 28°, the mycelium together with culture medium was first extracted three times with 10 l of a mixed solvent (80% AcOEt, 15% MeOH, and 5% AcOH). The crude extract was partitioned again with AcOEt (1 l) and H_2O (1 l). The org. layer was dried (Na_2SO_4), and the solvent was evaporated under reduced pressure to afford 12.6 g of a crude org. extract (brown solid). The crude extract was subjected to MPLC over *RP-18* SiO_2 (170 g), eluted with H_2O, then a stepwise gradient of 30, 50, 70, and 100% (*v*/*v*) MeOH in H_2O and to afford *Fr. 1* (621.2 mg) and *Fr. 2* (70.1 mg) obtained from 30% MeOH, *Fr. 3* (1.0433 g) obtained from 50% MeOH, and *Fr. 4* (1.073 g) and *Fr. 5* (858.2 mg) obtained from 70% MeOH. *Fr. 1* was further subjected to *Sephadex LH-20* eluted with acetone to afford *Fr. 1a* (17.8 mg), *Fr. 1b* (10.4 mg), and *Fr. 1c* (11.1 mg), which were subjected to SiO_2 chromatography using the same gradient of $CHCl_3$/MeOH (100 : 0, 100 : 1, 100 : 10) to yield **1** (12.5 mg), **2** (6.3 mg), and **3** (7.4 mg), resp. *Fr. 2* was subjected to CC (*Sephadex LH-20*; MeOH; and SiO_2; petroleum ether (PE)/$CHCl_3$ 8 : 1) to yield **8** (6.8 mg). *Fr. 3* was further purified by CC (SiO_2; PE/$CHCl_3$ 8 : 1) to yield **4** (347.2 mg). *Fr. 4* was purified by CC (SiO_2; PE/$CHCl_3$ 1 : 1) to yield **7** (42.0 mg); then it was further purified by CC (SiO_2; PE/$CHCl_3$ 3 : 4) to yield **5** (4.5 mg). *Fr. 5* was further purified by CC (SiO_2; PE/$CHCl_3$ 10 : 1) to yield **6** (16.1 mg).

Biological Studies. Cytotoxicities of compounds **1**–**8** were investigated using the human cancer cell lines HeLa, HepG-2, and A-549, following the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) standards [20], and cisplatin (DDP) was used as a positive control in this experiment. Further, the antibacterial activities of **1**–**8** were tested against four bacteria (*Bacillus subtilis*, *Bacillus Pumilus*, *Escherichia coli*, and *Staphylococcus aureus*) and two yeasts (*Candida albicans* and *Aspergillus niger*) by the minimal-inhibitory concentration (*MIC*) method [21] using 96-well microplates. Three replicates were performed for each compound.

Botryosphaerone A (= *(3S*,4S*)-3,4-Dihydro-3,4,8-trihydroxy-7-(1-hydroxyethyl)-6-methoxynaphthalen-1(2H)-one*; **1**). White solid. $[\alpha]_D^{20} = +18.5$ (*c* = 2.0, MeOH). UV (MeOH): 230 (3.46), 283 (3.93). IR (KBr): 3369, 1622, 1294, 1074. ^{1}H- and ^{13}C-NMR: see *Tables 2* and *1*, resp. HR-Q-TOF-MS: 291.0840 ($[M+Na]^+$, $C_{13}H_{16}NaO_6^+$; calc. 291.0845).

Botryosphaerone B (= *(3S*,4S*)-3,4-Dihydro-3,4,8-trihydroxy-7-(2-hydroxyethyl)-6-methoxynaphthalen-1(2H)-one*; **2**). White solid. $[\alpha]_D^{20} = +7.5$ (*c* = 2.0, MeOH). UV (MeOH): 230 (2.77), 287 (3.00).

IR (KBr): 3312, 1623, 1298, 1288, 1013. ^{1}H- and ^{13}C-NMR: see *Tables 2* and *1*, resp. HR-Q-TOF-MS: 291.0848 ($[M+Na]^+$, $C_{13}H_{16}NaO_6^+$; calc. 291.0845).

Botryosphaerone C (=*(3S*,4S*)-3,4-Dihydro-3,4,8-trihydroxy-6-methoxy-7-(1-methoxyethyl)naphthalen-1(2H)-one*; **3**). White solid. $[\alpha]_D^{20} = +18.0$ ($c = 2.0$, MeOH). UV (MeOH): 230 (2.47), 292 (2.87). IR (KBr): 3384, 1621, 1293, 1119, 1077. ^{1}H- and ^{13}C- NMR: see *Tables 2* and *1*, resp. HR-Q-TOF-MS: 305.1005 ($[M+Na]^+$, $C_{14}H_{18}NaO_6^+$; calc. 305.1001).

Botryosphaerone D (=*(3S*,4S*)-7-Ethyl-3,4-dihydro-3,4,8-trihydroxy-6-methoxynaphthalen-1(2H)-one*; **4**). White solid. $[\alpha]_D^{20} = +13.0$ ($c = 2.0$, MeOH). UV (MeOH): 230 (2.19), 297 (2.78). IR (KBr): 3428, 1626, 1311. ^{1}H- and ^{13}C-NMR: see *Tables 2* and *1*, resp. HR-Q-TOF-MS: 275.0900 ($[M+Na]^+$, $C_{13}H_{16}NaO_5^+$; calc. 275.0895).

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