Exserolides A–F, new isocoumarin derivatives from the plant endophytic fungus *Exserohilum* sp.

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ABSTRACT

Six new isocoumarin derivatives, exserolides A–F (**1**–**6**), were isolated from solid cultures of the plant endophytic fungus *Exserohilum* sp., together with four known metabolites (**7**–**10**). The structures of **1**–**6** were elucidated primarily by NMR experiments. The absolute configuration of the C-3 methine carbon in **1**–**5** was deduced via the circular dichroism data, whereas that of the 1,3-diol moiety in **6** was assigned from the ¹H NMR data of its (*R*)- and (*S*)-MTPA diesters. Compounds **3** and **9** showed antifungal activity against the plant pathogen *Fusarium oxysporum*, whereas **6** displayed significant inhibitory effects against a small panel of bacteria.

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1. Introduction

Endophytic fungi are well-known as a rich source of bioactive natural products [1,2]. Although the species of the fungal genus *Exserohilum* are very common in their distribution, the chemistry of this class of fungi remained largely un-explored. To date, only a few bioactive secondary metabolites have been isolated from *Exserohilum* spp., including exserohilone [3], rostratins A–D [4],

and 11-hydroxymonocerin [5]. During an ongoing search for new bioactive natural products from the plant endophytic fungi [6–10], a strain of *Exserohilum* sp. isolated from the leaves of *Acer truncatum* Bunge that were collected from Dongling Mountain, Beijing, People's Republic of China, was subjected to chemical investigation. An EtOAc extract prepared from solid–substrate fermentation products of the fungus showed antimicrobial activity against a small panel of bacteria including *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (CGMCC 1.2465), *Streptococcus pneumoniae* (CGMCC 1.1692), and *Escherichia coli* (CGMCC 1.2340), as well as the plant pathogenic fungus *Fusarium oxysporum* (CGMCC 3.2830). Bioassay-directed

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fractionation of the extract led to the isolation of six new isocoumarin derivatives, named exserolides A–F (**1**–**6**), and four known compounds, monocerin (**7**) [11,12], 11-hydroxymonocerin (**8**) [5], (12*R*)- (**9**) and (12*S*)-12-hydroxymonocerin (**10**) [13]. Details of the isolation, structure elucidation, and antimicrobial activities of these new compounds are reported herein.

2. Experimental

2.1. General

Optical rotations were measured on a Perkin-Elmer 241 polarimeter, and UV data were recorded on a Shimadzu Biospec-1601 spectrophotometer. CD spectra were recorded on a JASCO J-815 spectropolarimeter. IR data were recorded using a Nicolet Magna-IR 750 spectrophotometer. ^{1}H and ^{13}C NMR data were acquired with a Bruker Avance-600 spectrometer using solvent signals (CDCl$_3$: δ_H 7.26/δ_C 77.2; acetone-d_6: δ_H 2.05/δ_C 29.8, 206.1) as references. The HMQC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. HRESIMS data were obtained using an Agilent Accurate-Mass-Q-TOF LC/MS 6520 instrument equipped with an electrospray ionization (ESI) source. The fragmentor and capillary voltages were kept at 125 and 3500 V, respectively. Nitrogen was supplied as the nebulizing and drying gas. The temperature of the drying gas was set at 300 °C. The flow rate of the drying gas and the pressure of the nebulizer were 10 L/min and 10 psi, respectively. All MS experiments were performed in positive ion mode. Full-scan spectra were acquired over a scan range of *m/z* 100–1000 at 1.03 spectra/s.

2.2. Fungal material

The culture of *Exserohilum* sp. was isolated from the leaves of *A. truncatum* Bunge, collected from Dongling Mountain, Beijing, People's Republic of China, in August, 2007. The isolate was identified by one of the authors (L.G.) on the basis of morphology and sequence (GenBank accession No. KJ156361) analysis of the ITS region of the rDNA. The fungal strain was cultured on slants of potato dextrose agar (PDA) at 25 °C for 10 days. Agar plugs were cut into small pieces (about 0.5 × 0.5 × 0.5 cm^3) under aseptic conditions, and 15 pieces were used to inoculate three Erlenmeyer flasks (250 mL), each containing 50 mL of media (0.4% glucose, 1% malt extract, and 0.4% yeast extract; the final pH of the media was adjusted to 6.5 and sterilized by autoclave). Three flasks of the inoculated media were incubated at 25 °C on a rotary shaker at 170 rpm for five days to prepare the seed culture. Fermentation was carried out in 12 Fernbach flasks (500 mL), each containing 80 g of rice. Distilled H$_2$O (120 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 15 psi for 30 min. After cooling to room temperature, each flask was inoculated with 5.0 mL of the spore inoculum and incubated at 25 °C for 40 days.

2.3. Extraction and isolation

The fermented material was extracted repeatedly with EtOAc (4 × 1.0 L), and the organic solvent was evaporated to dryness under vacuum to afford the crude extract (13.0 g),

which was fractionated by silica gel VLC using petroleum ether–CH$_2$Cl$_2$–MeOH gradient elution. The fraction (255 mg) eluted with 100:1 CH$_2$Cl$_2$–MeOH was purified by semi-preparative RP HPLC (Agilent Zorbax SB-C$_{18}$ column; 5 μm; 9.4 × 250 mm; 70% MeOH in H$_2$O over 20 min; 2 mL/min) to afford **7** (103.0 mg, t_R 11.45 min). The fraction (2.0 g) eluted with 100:1.5 CH$_2$Cl$_2$–MeOH was separated by silica gel column using petroleum ether–EtOA–MeOH gradient elution to yield 10 fractions (Fr. 1–Fr. 10). Fr. 6 was further purified by semi-preparative RP HPLC (60% MeOH in H$_2$O for 30 min; 2 mL/min) to afford **1** (3.4 mg, t_R 23.5 min) and **4** (7.5 mg, t_R 15.2 min). The fraction (484 mg) eluted with 100:2 CH$_2$Cl$_2$–MeOH was separated by Sephadex LH-20 column chromatography (CC) eluting with 1:1 CH$_2$Cl$_2$–MeOH. The resulting subfractions were further purified by semipreparative RP HPLC (42% MeOH in H$_2$O for 40 min; 2 mL/min) to afford **10** (1.9 mg, t_R 21.0 min), **8** (2.6 mg, t_R 26.0 min), and **5** (2.5 mg, t_R 29.7 min). The fraction (355 mg) eluted with 100:2.5 CH$_2$Cl$_2$–MeOH was separated by Sephadex LH-20 CC eluting with 1:1 CH$_2$Cl$_2$–MeOH. The subfractions were combined and further purified by RP HPLC (44% MeOH in H$_2$O for 30 min, followed by 44–70% MeOH over 30 min; 2 mL/min) to afford **9** (5.3 mg, t_R 24.5 min), **2** (10.3 mg, t_R 28.5 min), and **3** (11.0 mg, t_R 36.0 min). While the fraction (110 mg) eluted with 100:4 CH$_2$Cl$_2$–MeOH was purified by RP HPLC (62% MeOH in H$_2$O over 30 min; 2 mL/min) to afford **6** (3.0 mg, t_R 14.9 min).

2.3.1. Exserolide A (1)

Colorless oil, $[\alpha]^{25}_D$ +66.7 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε): 223 (3.47), 273 (3.20), 306 (2.74) nm; CD (*c* 1.6 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 213 (+7.98), 271 (−4.47); IR (neat) ν_{max} 3320 (br), 2959, 1666, 1465, 1379, 1274, 1119, 849 cm^{-1}; ^{1}H, ^{13}C NMR, and HMBC data see Table 1; HRESIMS *m/z* 309.1334 [M + H]$^+$ (calcd for C$_{16}$H$_{21}$O$_6$, 309.1333).

2.3.2. Exserolide B (2)

White powder, $[\alpha]^{25}_D$ +7.3 (*c* 0.45, MeOH); UV (MeOH) λ_{max} (log ε): 219 (3.41), 271 (3.16), 307 (2.66) nm; CD (*c* 1.2 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 218 (+4.41), 270 (−4.78); IR (neat) ν_{max} 3384 (br), 2953, 1636, 1410, 1284, 1126, 1023 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; HMBC data (CDCl$_3$, 150 MHz) H-3 → C-4, 10; H-4 → C-3, 4a, 5, 8a; H-5 → C-4, 6, 7, 8a; H$_2$-9 → C-3, 4, 11; H$_3$-13 → C-11, 12; H$_3$-14 → C-7; H$_3$-15 → C-6; 8-OH → C-7, 8, 8a; HRESIMS *m/z* 309.1336 [M + H]$^+$ (calcd for C$_{16}$H$_{21}$O$_6$, 309.1333).

2.3.3. Exserolide C (3)

White powder, $[\alpha]^{25}_D$ −17.1 (*c* 0.65, MeOH); UV (MeOH) λ_{max} (log ε): 220 (3.52), 271 (3.26), 308 (2.74) nm; CD (*c* 1.7 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 216 (+4.83), 270 (−4.66); IR (neat) ν_{max} 3415 (br), 2957, 1667, 1455, 1282, 1126 cm^{-1}; ^{1}H and^{13}C NMR data see Table 1; HMBC data (CDCl$_3$, 150 MHz) H-3 → C-4, 10; H-4 → C-3, 4a, 5, 8a; H-5 → C-4, 6, 7, 8a; H$_2$-9 → C-3, 4, 11; H$_3$-13 → C-11, 12; H$_3$-14 → C-7; H$_3$-15 → C-6; 8-OH → C-7, 8, 8a; HRESIMS *m/z* 309.1335 [M + H]$^+$ (calcd for C$_{16}$H$_{21}$O$_6$, 309.1333).

2.3.4. Exserolide D (4)

Colorless oil, $[\alpha]^{25}_D$ +40.5 (*c* 0.21, MeOH); UV (MeOH) λ_{max} (log ε): 218 (3.31), 273 (3.03), 304 (2.70) nm; CD (*c* 1.0 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 210 (+7.41), 271 (−4.11);

Table 1
NMR data for **1**–**3**.

Pos	**1** $\delta_C{}^a$, mult.	$\delta_H{}^b$ (J in Hz)	HMBC^a	**2** $\delta_C{}^a$, mult.	$\delta_H{}^b$ (J in Hz)	**3** $\delta_C{}^a$, mult.	$\delta_H{}^b$ (J in Hz)
1	168.2, qC			168.9, qC		169.3, qC	
2							
3	82.0, CH	5.14, m	4, 10	79.6, CH	4.74, ddd (8.4, 5.4, 1.8)	79.8, CH	4.71, ddd (7.8, 5.4, 1.8)
4	73.6, CH	4.80, d (3.0)	3, 4a, 5, 8a	67.1, CH	4.66, d (1.8)	66.6, CH	4.64, d (1.8)
4a	132.1, qC			136.5, qC		136.8, qC	
5	104.4, CH	6.57, s	4, 6, 7, 8a	103.0, CH	6.56, s	103.1, CH	6.54, s
6	159.0, qC			159.0, qC		159.0, qC	
7	137.4, qC			137.0, qC		137.0, qC	
8	156.5, qC			156.2, qC		156.2, qC	
8a	101.7, qC			102.1, qC		102.0, qC	
9	40.1, CH$_2$	2.61, ddd (13.8, 5.4, 0.6); 2.00, ddd (13.8, 9.6, 4.2)	3, 4, 11	37.8, CH$_2$	2.17, ddd (15.0, 8.4, 1.8); 1.88, ddd (15.0, 10.2, 5.4)	37.7, CH$_2$	2.21, ddd (15.0, 7.8, 3.0); 2.04, ddd (15.0, 8.4, 5.4)
10	79.3, CH	4.38, m		68.0, CH	3.97, m	68.1, CH	4.04, m
11	38.4, CH$_2$	1.69, m; 1.54, m		40.7, CH$_2$	1.54, m; 1.49, m	39.8, CH$_2$	1.53, m; 1.48, m
12	19.3, CH$_2$	1.46, m; 1.38, m		18.8, CH$_2$	1.52, m; 1.40, m	18.9, CH$_2$	1.51, m; 1.38, m
13	14.2, CH$_3$	0.95, t (7.8)	11, 12	14.1, CH$_3$	0.96, t (7.2)	14.1, CH$_3$	0.95, t (7.2)
14	60.9, CH$_3$	3.89, s	7	61.0, CH$_3$	3.89, s	60.9, CH$_3$	3.87, s
15	56.5, CH$_3$	3.95, s	6	56.5, CH$_3$	3.94, s	56.4, CH$_3$	3.93, s
8-OH		11.25, s	7, 8, 8a		11.07, s		11.00, s

^{a} Recorded at 150 MHz in CDCl$_3$.
^{b} Recorded at 600 MHz in CDCl$_3$.

IR (neat) ν_{max} 3361 (br), 2959, 1662, 1461, 1380, 1288, 1110, 859 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 2; HMBC data (CDCl$_3$, 150 MHz) H-3 → C-4, 10; H-4 → C-3, 4a, 5, 8a; H-5 → C-4, 6, 7, 8a; H$_2$-9 → C-3, 4, 11; H$_3$-13 → C-11, 12; H$_3$-14 → C-7; 6-OH → C-5, 6, 7; 8-OH → C-7, 8, 8a; HRESIMS m/z 295.1184 [M + H]$^+$ (calcd for C$_{15}$H$_{19}$O$_6$, 295.1176).

Table 2
NMR data for **4**–**6**.

Pos	**4** $\delta_C{}^a$, mult.	$\delta_H{}^b$ (J in Hz)	**5** $\delta_C{}^a$, mult.	$\delta_H{}^b$ (J in Hz)	**6** $\delta_C{}^c$, mult.	$\delta_H{}^d$ (J in Hz)
1	168.2, qC		167.8, qC		167.0, qC	
2						
3	81.5, CH	5.03, ddd (6.0, 3.0, 0.6)	80.9, CH	5.10, m	156.5, qC	
4	74.4, CH	4.49, d (3.0)	74.8, CH	4.60, d (3.0)	106.4, CH	6.44, s
4a	131.5, qC		130.8, qC		140.9, qC	
5	108.0, CH	6.61, s	104.5, CH	6.59, s	103.2, CH	6.41, d (1.8)
6	155.3, qC		158.9, qC		166.3, qC	
7	135.0, qC		137.7, qC		102.1, CH	6.37, d (1.8)
8	155.6, qC		156.5, qC		164.3, qC	
8a	101.5, qC		102.2, qC		99.8, qC	
9	39.2, CH$_2$	2.57, ddd (14.4, 8.4, 6.0); 2.14, ddd (14.4, 6.0, 0.6)	34.0, CH$_2$	2.52, ddd (14.4, 6.6, 1.8); 2.48, ddd (14.4, 8.4, 6.0)	42.8, CH$_2$	2.67, dd (14.4, 4.8); 2.60, dd (14.4, 8.4)
10	78.8, CH	4.11, m	81.6, CH	4.08, m	66.6, CH	4.32, m
11	38.2, CH$_2$	1.69, m; 1.57, m	73.2, CH	3.72, m	44.8, CH$_2$	1.60, m
12	19.3, CH$_2$	1.42, m; 1.35, m	26.1, CH$_2$	1.54, m; 1.43, m	68.2, CH	3.90, m
13	14.1, CH$_3$	0.91, t (7.8)	10.2, CH$_3$	0.98, t (7.8)	41.2, CH$_2$	1.45, m; 1.40, m
14	61.0, CH$_3$	4.00, s	60.9, CH$_3$	3.91, s	19.5, CH$_2$	1.46, m; 1.36, m
15			56.5, CH$_3$	3.96, s	14.4, CH$_3$	0.90, t (7.2)
6-OH		6.37, s				9.63, s
8-OH		11.52, s		11.28, s		11.16, s
10-OH						4.12, d (4.8)
12-OH						3.63, d (5.4)

^{a} Recorded at 150 MHz in CDCl$_3$.
^{b} Recorded at 600 MHz in CDCl$_3$.
^{c} Recorded at 150 MHz in acetone-d_6.
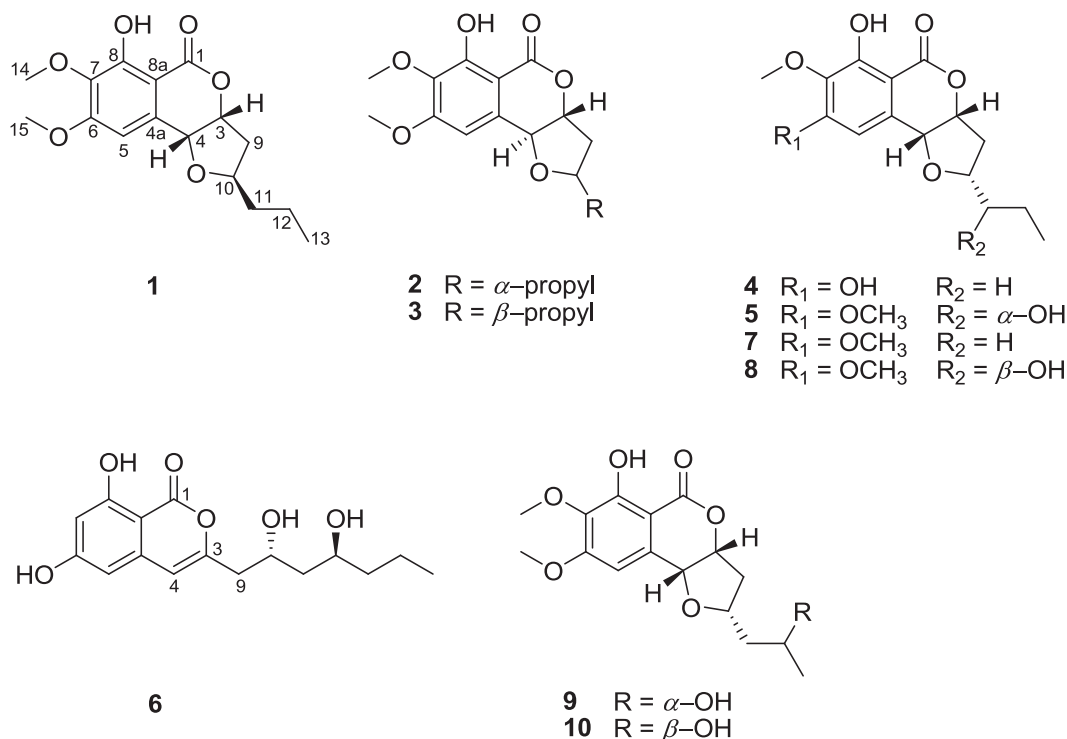^{d} Recorded at 600 MHz in acetone-d_6.

Fig. 1. Structures of compounds 1–10.

2.3.5. Exserolide E (5)

Colorless oil, $[\alpha]^{25}_D$ +36.6 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε): 222 (3.50), 271 (3.22), 306 (2.74) nm; CD (c 5.0 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 215 (+2.64), 271 (−1.84); IR (neat) ν_{max} 3432 (br), 2964, 1666, 1456, 1391, 1277, 1123, 1031 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 2; HMBC data (CDCl$_3$, 150 MHz) H-3 → C-4, 10; H-4 → C-3, 4a, 5, 8a; H-5 → C-4, 6, 7, 8a; H$_2$-9 → C-3, 4, 11; H$_3$-13 → C-11, 12; H$_3$-14 → C-7; H$_3$-15 → C-6; 8-OH → C-7, 8, 8a; HRESIMS *m/z* 325.1278 [M + H]$^+$ (calcd for C$_{16}$H$_{21}$O$_7$, 325.1282).

2.3.6. Exserolide F (6)

Light yellow powder, $[\alpha]^{25}_D$ −15.6 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε): 237 (2.99), 244 (3.06), 276 (2.21), 289 (2.07), 326 (2.20) nm; IR (neat) ν_{max} 3334 (br), 3149, 2961, 1678, 1629, 1467, 1386, 1171, 1069, 843 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 2; HMBC data (acetone-d_6, 150 MHz) H-4 → C-3, 4a, 5, 8a, 9; H-5 → C-4, 6, 7, 8a; H-7 → C-5, 6, 8, 8a; H$_2$-9 → C-3, 4, 10, 11; H$_2$-11 → C-9, 10, 12, 13; H$_3$-15 →

C-13, 14; 6-OH → C-5; 8-OH → C-7, 8, 8a; 10-OH → C-9, 10, 11; 12-OH → C-11, 12, 13; HRESIMS *m/z* 331.1155 [M + Na]$^+$ (calcd for C$_{16}$H$_{20}$NaO$_6$, 331.1152).

2.3.7. Preparation of (R)-(6a) and (S)-MTPA (6b) esters

A sample of **6** (0.6 mg, 0.002 mmol) was dissolved in 500 μL anhydrous pyridine in a 5 mL vial. (*S*)-MTPA Cl (5.0 μL, 0.028 mmol) was quickly added, the vial was sealed, and the mixture was kept at ambient temperature for 12 h. The mixture was evaporated to dryness and purified by RP HPLC (Agilent Zorbax SB-C$_{18}$ column; 5 μm; 9.4 × 250 mm; 92% MeOH in H$_2$O for 20 min; 2 mL/min) to afford the MTPA ester **6a** (0.8 mg, t_R 10.5 min): colorless oil; ^{1}H NMR (acetone-d_6, 600 MHz) δ 7.43 (1H, d, *J* = 1.8 Hz, H-5), 7.20 (1H, d, *J* = 1.8 Hz, H-7), 6.58 (1H, s, H-4), 5.38 (1H, m, H-10), 5.07 (1H, m, H-12), 3.00 (1H, dd, *J* = 15.0, 6.6 Hz, H-9a), 2.95 (1H, dd, *J* = 15.0, 6.0 Hz, H-9b), 2.12 (1H, m, H-11a), 2.06 (1H, m, H-11b), 1.69 (1H, m, H-13a), 1.60 (1H, m, H-13b), 1.29 (1H, m, H-14a), 1.29 (1H, m, H-14b), 0.85 (3H, t, *J* = 7.8 Hz, H-15).
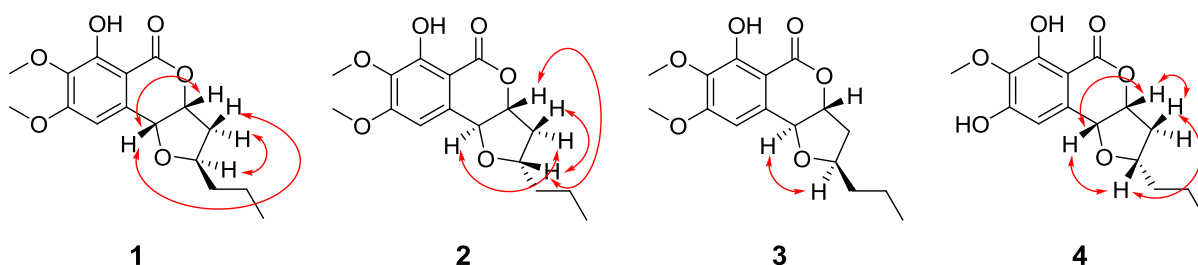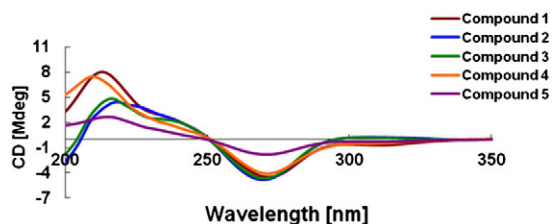

Fig. 2. Key NOESY correlations of 1–4.

Fig. 3. CD spectra of 1–5 in MeOH.

In a similar fashion, a sample of **6** (0.6 mg, 0.002 mmol), (*R*)-MTPA Cl (5.0 μL, 0.028 mmol) and anhydrous pyridine (500 μL) were allowed to react in a 5 mL vial at ambient temperature for 12 h, and the reaction mixture was processed as described above for **6a** to afford the MTPA ester **6b** (0.9 mg, t_R 10.6 min): colorless oil; ^{1}H NMR (acetone-d_6, 600 MHz) δ 7.36 (1H, d, *J* = 1.8 Hz, H-5), 7.18 (1H, d, *J* = 1.8 Hz, H-7), 6.48 (1H, s, H-4), 5.61 (1H, m, H-10), 5.24 (1H, m, H-12), 3.00 (1H, overlap, H-9a), 2.99 (1H, overlap, H-9b), 2.24 (1H, m, H-11a), 2.24 (1H, m, H-11b), 1.66 (1H, m, H-13a), 1.63 (1H, m, H-13b), 1.27 (1H, m, H-14a), 1.20 (1H, m, H-14b), 0.84 (3H, t, *J* = 7.2 Hz, H-15).

2.4. Antibacterial and antifungal assays

Antibacterial and Antifungal bioassays were conducted in triplicate following the National Center for Clinical Laboratory Standards (NCCLS) recommendations [14,15]. The bacterial strains *B. subtilis* (ATCC 6633), *S. aureus* (CGMCC 1.2465), *S. pneumoniae* (CGMCC 1.1692), and *E. coli* (CGMCC 1.2340), were grown on Mueller–Hinton agar. The plant pathogenic fungus, *F. oxysporum* (CGMCC 3.2830), was grown on potato dextrose agar. Targeted microbes (3 or 4 colonies) were prepared from broth cultures (bacteria: 37 °C for 24 h; fungus: 28 °C for 48 h), and the final spore suspensions of bacteria (in MHB medium), and the plant pathogenic fungus (in PDB medium) were 10^6 cells/mL and 10^4 mycelial fragments/mL, respectively. Test samples (10 mg/mL as stock solution in DMSO and serial dilutions) were transferred to 96-well clear plates in triplicate, and the suspensions of the test organisms were added to each well, achieving a final volume of 200 μL. After incubation (bacteria: 37 °C for 24 h; fungus: 28 °C for 48 h), the fluorescence intensity was measured at Ex/Em = 544/590 nm using a microtiter plate reader. The inhibition rates were calculated and plotted versus the test concentrations to afford the MICs, which were defined as the lowest concentration that completely inhibited the growth of the test organisms.

3. Results and discussion

Exserolide A (**1**) was assigned the molecular formula $C_{16}H_{20}O_6$ (seven degrees of unsaturation) on the basis of HRESIMS (*m/z* 309.1334 [M + H]$^+$; Δ − 0.1 mmu). Its ^{1}H and ^{13}C NMR spectra showed resonances for one exchangeable proton ($δ_H$ 11.25) and three methyl groups including two *O*-methyls, three methylenes, three *O*-methines, six aromatic carbons (one of which was protonated), and one carboxylic carbon ($δ_C$ 168.2). Analysis of the NMR data of **1** (Table 1) revealed the presence of the same 8-hydroxyisochroman-

1-one moiety as that typically found in monocerin (**7**) [11,12] In addition to the above-mentioned fragment, the ^{1}H–^{1}H COSY NMR data of **1** showed one isolated spin-system of C-4–C-3–C-9–C-13. HMBC correlations from the *O*-methyl proton signals H_3-14 to C-7, and H_3-15 to C-6 located the two *O*-methyl groups at C-7 and C-6, respectively. Considering the chemical shifts of the C-4 and C-10 *O*-methines ($δ_H$/$δ_C$ 4.80/73.6, 4.38/79.3, respectively) and the unsaturation requirement of **1**, C-4 and C-10 are now attached to the remaining oxygen atom to form a tetrahydrofuran (THF) ring fused to the 8-hydroxyisochroman-1-one moiety via C-3–C-4, completing the gross structure of **1** as shown (Fig. 1).

The relative configuration of **1** was proposed by analysis of NOESY data and by comparison with that of a model compound, lasionectrin [16]. NOESY correlations of H-4 with H-3 and H-9b placed these protons on the same face of the THF ring, whereas that of H-10 with H-9a revealed their proximity in space, thereby allowing the deduction of the relative configuration for **1** (Fig. 2).

The absolute configuration of the C-3 methine carbon in **1** was deduced by the application of the circular dichroism (CD) exciton chirality method. The CD spectrum of **1** showed a negative Cotton effect at 270 nm (Δε− 4.47) (Fig. 3), similar to that observed in the model compounds (−)-mellein and (*R*)-5-chloro-6-hydroxymellein [17,18], allowing assignment of the 3*R* absolute configuration. Therefore, the absolute configuration of **1** was deduced to be 3*R*, 4*R*, 10 *R*.

Exserolide B (**2**) was assigned the same molecular formula $C_{16}H_{20}O_6$ (seven degrees of unsaturation) as **1** by HRESIMS (*m/z* 309.1336 [M + H]$^+$; Δ –0.3 mmu). Interpretation of its ^{1}H and ^{13}C NMR spectroscopic data (Table 1) revealed the same planar structure as **1**, which was supported by relevant ^{1}H–^{1}H COSY and HMBC data, suggesting that **2** is a stereoisomer of **1**. The relative configuration of **2** was deduced by the analysis of ^{1}H–^{1}H coupling constants and NOESY data. A coupling constant of 1.8 Hz observed between H-3 and H-4, compared to 1.0 Hz for the same protons in a model compound arvensin [19], together with the NOESY correlations of H-9a with H-4, H-9b with H-10, and H-3 with H-10, indicating that these protons are on the same face of the THF ring. Thereby, the relative configuration of **2** was assigned (Fig. 2). The absolute configuration of the C-3 methine carbon in **2** was also deduced via the CD data. The CD spectrum of **2** showed a negative Cotton effect at 270 nm (Δε –4.78) (Fig. 3), correlating to the 3*R* absolute configuration [17,18].
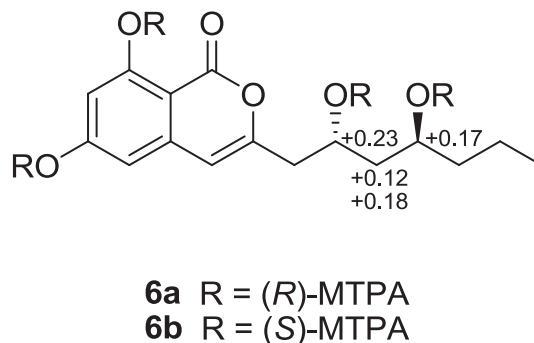

6a R = (*R*)-MTPA
6b R = (*S*)-MTPA

Fig. 4. Δδ values (in ppm) = $δ_S − δ_R$ obtained for (*R*)- and (*S*)-MTPA diesters 6a and 6b.

Therefore, the 3*R*, 4*S*, 10*S* absolute configuration was proposed for **2**.

The elemental composition of exserolide C (**3**) was established as $C_{16}H_{20}O_6$ (seven degrees of unsaturation) by HRESIMS (*m/z* 309.1335 [M + H]$^+$; Δ −0.2 mmu), which is the same as **1** and **2**. Interpretation of the 2D NMR data of **3** established the same gross structure as **1** and **2**, indicating their isomeric relationships.

The relative configuration of **3** was assigned by the analysis of its ^1H–^1H coupling constants and NOESY data. The same coupling constant of 1.8 Hz observed between H-4 and H-3 in **3** and **2** revealed a *trans* relationship for the two protons, which was supported by the NMR data (the chemical shift values for C-3, C-4, and C-4a are nearly identical in both compounds; Table 1). A NOESY correlation of H-4 with H-10 placed these two protons on the same face of the THF ring system, permitting the assignment of the relative configuration of **3** (Fig. 2). The CD spectrum of **3** showed a negative Cotton effect at 270 nm (Δε −4.66) (Fig. 3), correlating to the 3*R* absolute configuration [17,18]. Therefore, the absolute configuration of **3** was deduced to be 3*R*, 4*S*, 10 *R*.

Exserolide D (**4**) gave a pseudomolecular ion [M + H]$^+$ peak by HRESIMS (*m/z* 295.1184 [M + H]$^+$; Δ −0.8 mmu), establishing the molecular formula $C_{15}H_{18}O_6$ (seven degrees of unsaturation). Analysis of its NMR data (Table 2) revealed nearly identical structural features to those found in the known compound monocerin (**7**) [11,12], except that the *O*-methyl was replaced by a C-6 hydroxyl group (δ$_H$ 6.37) in **4**, which was confirmed by relevant ^1H–^1H COSY and HMBC data, indicating that **4** is an analog of **7**. The relative configuration of **4** was deduced to be the same as that of **7** by analysis of the ^1H–^1H coupling constants and NOESY data for relevant protons. The absolute configuration of **4** was also similarly deduced as shown via the CD data.

Exserolide E (**5**) was obtained as colorless oil. HRESIMS (*m/z* 325.1278 [M + H]$^+$; Δ +0.4 mmu) data for **5** gave the same molecular formula $C_{16}H_{20}O_7$ as a known compound 11-hydroxymonocerin (**8**) [5]. Its NMR data (Table 2) are nearly identical to those of **8**, except that the chemical shifts for the C-11 oxymethine (δ$_H$/δ$_C$ 3.72/73.2) were different from those in **8** (δ$_H$/δ$_C$ 3.53/75.0), indicating that **5** is an C-11 epimer of **8**. This postulation was supported by the 2D NMR and CD data.

The molecular formula of exserolide F (**6**) was determined to be $C_{16}H_{20}O_6$ (seven degrees of unsaturation) on the basis of its HRESIMS (*m/z* 331.1155 [M + Na]$^+$; Δ −0.3 mmu) data. Analysis of its ^1H and ^{13}C NMR data (Table 2) revealed the presence of four exchangeable protons (δ$_H$ 3.63, 4.12, 9.63, and 11.16, respectively), one methyl group, four methylenes, two *O*-methines, eight aromatic/olefinic carbons with three protonated, and one carboxylic carbon (δ$_C$ 167.0). Interpretation of its NMR data revealed the same 1,2,3,5-tetrasubstituted aryl ring with two hydroxy groups attached to C-6 and C-8, respectively, as appeared in (−)-citreoisocoumarinol [20] and citreoiswoumarinol [21]. The C-1 carboxylic carbon (δ$_C$ 167.0) was connected to C-8a based on the downfield ^1H NMR chemical shift for 8-OH (δ$_H$ 11.16). The ^1H–^1H COSY NMR data showed one isolated spin-system of C-9–C-15 (including 10-OH and 12-OH). HMBC correlations from H-4 to C-3, C-4a, C-5, C-8a, and C-9 and from H-9 to C-3 and C-4 connected C-4a to C-4 and C-3 to C-9, respectively. Considering the chemical shift values of C-1 (δ$_C$ 167.0) and C-3 (δ$_C$ 156.5) and the

unsaturation requirement for **6**, they were attached to the only remaining oxygen atom to complete the planar structure of **6** as shown (Fig. 1).

The absolute configuration of the 10,12-diol moiety in **6** was assigned from the ^1H NMR data its (*R*)- and (*S*)-MTPA diesters [22,23]. Treatment of **6** with (*S*)- and (*R*)-MTPA Cl afforded the *R*-MTPA (**6a**) and *S*-MTPA (**6b**) diesters, respectively. The difference in chemical shift values (Δδ = δ$_S$ − δ$_R$) for **6b** and **6a** was calculated to assign the 10*R* and 12*S* absolute configuration by applying the 1,3-*anti*-diol model as reported by Riguera et al. [22,23] (Fig. 4).

Compounds **7**–**10** were also isolated from the crude extract and identified as the known metabolites, monocerin (**7**) [11,12], 11-hydroxymonocerin (**8**) [5], and (12*R*)- (**9**) and (12*S*)-12-hydroxymonocerin (**10**) [13], respectively, by comparison of their NMR and MS data with those reported.

Compounds **1**–**10** were tested for their antimicrobial activity against a small panel of bacteria including *B. subtilis* (ATCC 6633), *S. aureus* (CGMCC 1.2465), *S. pneumoniae* (CGMCC 1.1692), and *E. coli* (CGMCC 1.2340), as well as the plant pathogenic fungus *F. oxysporum* (CGMCC 3.2830) (Table 3). Compounds **3** and **9** displayed antifungal activity against *F. oxysporum*, both showing a MIC value of 20 μg/mL (the positive control amphotericin B showed a MIC value of 0.63 μg/mL). While compound **6** exhibited significant antibacterial effects against not only the Gram-positive bacteria including *B. subtilis*, *S. aureus*, and *S. pneumoniae*, with MIC values of 20, 5, and 10 μg/mL, respectively, (the positive control ampicillin showed MIC values of 1.25, 0.16, and 10 μg/mL), respectively. While compound **6** exhibited significant antibacterial effects against not only the Gram-positive bacteria including *B. subtilis*, *S. aureus*, and *S. pneumoniae*, with MIC values of 20, 5, and 10 μg/mL, respectively, (the positive control ampicillin showed MIC values of 1.25, 0.16, and 10 μg/mL) but also the Gram-negative bacterium *E. coli*, with a MIC value of 20 μg/mL (the positive control gentamicin showed a MIC value of 2.5 μg/mL).

Isocoumarins are a class of natural lactones with high structural diversity, which gives the corresponding dihydro-isocoumarins through catalytic hydrogenation. Natural products with the 3,3a-dihydro-2*H*-furo[3,2-c]isochromen-5(9b*H*)-one moiety are relatively rare [5,11–13,24]. Although compound **1** was previously synthesized [25,26], this is the first demonstration of the natural occurrence of **1**. Compound **2** is a C-4 stereoisomer of monocerin (**7**) [11,12], whereas **3** is a stereoisomer of monocerin at both C-4 and C-10. Compound **4** is structurally related to **7**, but differs from the known analog by having a hydroxyl group attached to C-6 instead of a *O*-methyl group. Compound **5** is a C-11 stereoisomer of 11-hydroxymonocerin (**8**) [5]. In addition, compounds **2** and **3** represent the first example of isocoumarin derivatives possessing the *trans*-fused furobenzopyranones moiety. Compound **6** is structurally related to the known compounds (−)-citreoisocoumarinol [20] and citreoiswoumarinol [21], but differs by having a heptane-2,4-diol attached to C-3 rather than a pentane-2,4-diol moiety, in addition to different configurations at C-10 and C-12.

Table 3
Antimicrobial activities of compounds **3**, **6**, and **9**.

Compound	MIC (µg/mL)				
	B. subtilis	*S. aureus*	*S. pneumoniae*	*E. coli*	*F. oxysporum*
3					20
6	20	5	10	20	
9					20
Ampicillin	1.25	0.16	10		
Gentamicin				2.5	
Amphotericin B					0.63

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2014.04.013.

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