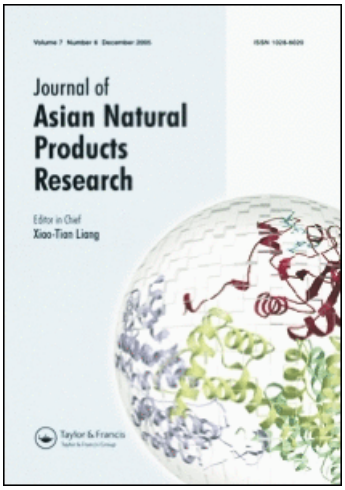

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ORIGINAL ARTICLE

Cochliones A–D, four new tetrahydrochromanone derivatives from endophytic *Cochliobolus* sp.

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Four new tetrahydrochromanone derivatives, cochliones A–D (**1**–**4**), along with three known metabolites, 4-hydroxybenzaldehyde (**5**), 4-hydroxy-3-(3-methylbut-2-enyl) benzoic acid (**6**), and 2,2-dimethyl-2*H*-chromene-6-carboxylic acid (**7**), were characterized from the culture of *Cochliobolus* sp. IFB-E039, a fungus that resides inside the healthy root of *Cynodon dactylon* (Gramineae). The structures of **1**–**4** were accommodated by their spectral data (UV, IR, CD, HR-ESI-MS, ^{1}H and ^{13}C NMR, ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY). The bioassay for the cytotoxic metabolite showed that cochlione C (**3**) inhibited human breast adenocarcinoma cell line (MCF-7) and human chronic myeloid leukemia cell line (K562) with IC_{50} values of 21.99 and 4.59 μg/ml, respectively.

Keywords: tetrahydrochromanone derivatives; cochliones A–D; *Cochliobolus* sp.; *Cynodon dactylon*; cytotoxicity

1. Introduction

Endophytic fungi, living inside the normal plant tissue without generating detectable symptoms, have been recognized as a big microbial reservoir, which was almost neglected a couple of decades ago. Owing to the presumable genetic recombination with host plants, some endophytes could be efficient generators of a wide variety of structurally unique and/or biologically potent natural products [1,2]. In continuation of our previous chemical and biological investigations of metabolites derived from symbiotic fungi [3,4], the presence of cytotoxin(s) was discerned in the fermentation broth of the endophytic fungal strain *Cochliobolus* sp. IFB-E039 that habituates inside the normal fresh root of *Cynodon dactylon* (Gramineae). The subsequent cytotoxicity-guided fractionation led to the isolation of four new tetrahydrochromanone derivatives that we have named as cochliones A–D (**1**–**4**) (Figure 1), coexisting in the fungal culture with 4-hydroxybenzaldehyde (**5**) [5], 4-hydroxy-3-(3-methylbut-2-enyl) benzoic acid (**6**) [6], and 2,2-dimethyl-2*H*-chromene-6-carboxylic acid (**7**) [7]. The structures of the new metabolites were elucidated by comprehensive spectroscopic analyses. We hereby wish to present the isolation, structural determination, and bioactivity assessment of these natural products.

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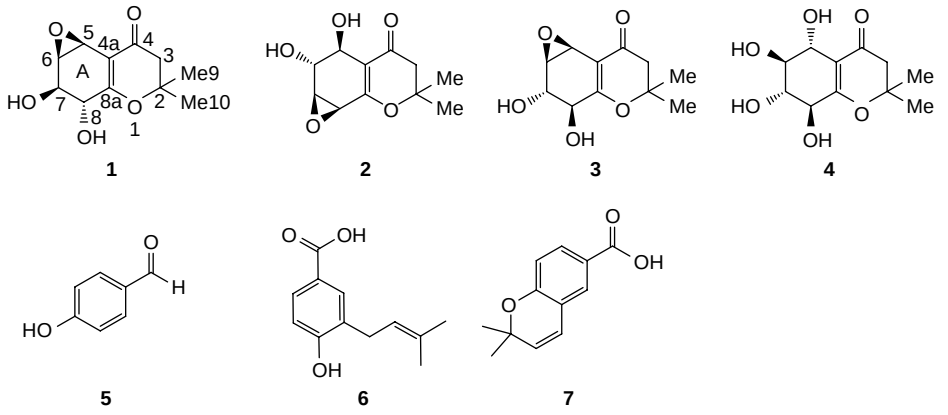

Figure 1. Structures of compounds **1**–**7**.

2. Results and discussion

Cautious fractionation of the extract derived from the liquid culture broth of *Cochliobolus* sp. IFB-E039 associated with the roots of a salinity-tolerant plant, *C. dactylon*, afforded four new tetrahydrochromanone derivatives, cochliones A–D (**1**–**4**), along with three known compounds, 4-hydroxybenzaldehyde (**5**), 4-hydroxy-3-(3-methylbut-2-enyl) benzoic acid (**6**), and 2,2-dimethyl-2*H*-chromene-6-carboxylic acid (**7**).

Cochlione A (**1**), obtained as a yellow amorphous powder, had a molecular formula of $C_{11}H_{14}O_5$ assigned unambiguously from its quasi-molecular ion ($[M + Na]^+$ at *m/z* 249.0731) in its high-resolution ESI mass spectrum. This formula retained five degrees of unsaturation, three of which were readily attributable to a double bond and an α,β-unsaturated ketone group as deduced from the ^{13}C NMR spectrum at δ 195.1, 106.0, and 166.0 ppm. The IR spectrum of compound **1** showed the absorption bands at 3395 and 1667 cm^{-1}, indicative of hydroxyl and ketone groups, respectively. Interpretation of the ^1H NMR (Table 1) and ^1H–^1H COSY spectra (Figure 2) indicated the presence of a four-proton coupling sequence that was significant at δ 3.41 (d, $J = 3.5$ Hz, H-5), 3.75 (dd,

Table 1. ^1H NMR spectral data of compounds **1**–**4** (δ, J in Hz)[a].

No.	1	2	3	4
3α	2.45 (d, $J = 16.5$)	2.50 (d, $J = 16.5$)	2.20 (d, $J = 16.5$)	2.55 (d, $J = 16.5$)
3β	2.67 (d, $J = 16.5$)	2.67 (d, $J = 16.5$)	2.46 (d, $J = 16.5$)	2.62 (d, $J = 16.5$)
5	3.41 (d, $J = 3.5$)	4.49 (br)	3.45 (d, $J = 3.5$)	4.65 (d, $J = 7.0$)
6	3.75 (dd, $J = 3.2$, 3.5)	4.68 (br)	3.75 (t, $J = 3.5$)	3.70 (dd, $J = 9.0$, 7.0)
7	4.45 (dd, $J = 4.5$, 3.2)	3.86 (br)	4.56 (t, $J = 3.5$)	3.84 (dd, $J = 8.0$, 9.0)
8	4.62 (d, $J = 4.5$)	3.53 (d, $J = 3.5$)	4.45 (br)	4.48 (d, $J = 8.0$)
9	1.44 (s)	1.41 (s)	1.29 (s)	1.46 (s)
10	1.52 (s)	1.52 (s)	1.33 (s)	1.49 (s)
5-OH		2.56 br s		3.53 br s
6-OH		4.13 br s		3.57 br s
7-OH	5.84 s		14.28 s	4.53 br s
8-OH	3.19 br s		2.47 br s	3.55 br s

Note: [a] Measured in $CDCl_3$ at 500 MHz.

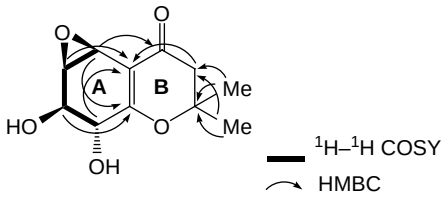

Figure 2. ^{1}H–^{1}H COSY and selected HMBC correlations of compound **1**.

$J = 3.2$, 3.5 Hz, H-6), 4.45 (dd, $J = 3.2$, 4.5 Hz, H-7), and 4.62 (d, $J = 4.5$ Hz, H-8). However, the magnitude of the corresponding oxymethine carbon resonance lines deviated strikingly in its ^{13}C NMR spectrum, suggesting the presence of an oxirane group [δ 49.5 (C-5) and 54.2 (C-6)] and two hydroxyl-methine carbons [δ 65.1 (C-7) and 65.6 (C-8)] [8–12] (Table 2). This assumption was confirmed by a set of 2D NMR experiments that provided decisive information concerning the structure of **1**. Thus, the HMBC correlations of H-5 with C-4 (δ 195.1) and C-8a (δ 166.0), and of H-8 with C-4a (δ 106.6) revealed that C-5 and C-8 were bridged through the 4a,8a-double bond involved in an α,β-unsaturated ketone moiety. Furthermore, the chemical shifts (δ_C 47.1 and δ_H 2.45 and 2.67) and larger coupling constant ($^2J = 16.5$ Hz) of the 3-methylene protons indicated that it was isolated by carbonyl and quaternary carbons. This was also reinforced by the HMBC correlations of H-3 with C-4 and C-4a. Furthermore, the HMBC correlations of H-9 and H-10 with C-2 and C-3 indicated that the 9,10-dimethyl groups were co-anchored on C-2. Finally, the relatively low-field resonances of sp^3-C-2 (δ 81.9) and sp^2-C-8a indicated that these two carbons were linked through an oxygen atom to form a 2,2-dimethyl-2*H*-pyran-4(3*H*)-one moiety. Concerning the relative configuration, the H-C(5), H-C(6), H-C(7), and H-C(8) in the structure of **1** were evidenced to be α-, α-, α-, and β-oriented, respectively, by the NOE correlations of H-6 with H-5 and H-7. However, the absolute configuration of this and other new compounds failed to be determined owing to the paucity of the material.

Cochlione B (**2**) also had a molecular formula of $C_{11}H_{14}O_5$ that was disclosed by its positive-ion HR-ESI-MS spectrum ($[M + Na]^+$ at *m/z* 249.0730), with its ^{1}H and ^{13}C NMR data being quite close to those of **1**. Furthermore, the ^{1}H NMR and ^{1}H–^{1}H COSY spectra of **2** again revealed a coupling sequence of four oxymethine protons including H-5 (δ 4.49), H-6 (δ 4.68), H-7 (δ 3.86), and H-8 (δ 3.53), indicative of the 7,8-epoxide. This assumption was affirmed by the HMBC correlations of H-8 with C-4a (δ 109.9) and C-8a (δ 167.1), and H-5 with C-4 (δ

Table 2. ^{13}C NMR spectral data of compounds **1**–**4**.

Position	**1**	**2**	**3**	**4**
2	81.9 (s)	82.5 (s)	72.0 (s)	82.1 (s)
3	47.1 (t)	47.1 (t)	40.9 (t)	47.0 (t)
4	195.1 (s)	191.3 (s)	191.8 (s)	194.4 (s)
5	49.5 (d)	66.6 (d)	53.3 (d)	69.3 (d)
6	54.2 (d)	64.6 (d)	54.8 (d)	74.2 (d)
7	65.1 (d)	58.0 (d)	65.8 (d)	74.0 (d)
8	65.6 (d)	49.8 (d)	65.6 (d)	59.1 (d)
9	24.0 (q)	25.0 (q)	24.2 (q)	25.3 (q)
10	27.7 (q)	27.2 (q)	30.1 (q)	26.5 (q)
4a	106.6 (s)	109.9 (s)	102.3 (s)	110.7 (s)
8a	166.0 (s)	167.1 (s)	178.2 (s)	162.0 (s)

Note: a, measured in $CDCl_3$ at 125 MHz.

Table 3. The *in vitro* cytotoxicity (IC_{50} μg/ml) of compounds **1**–**4** against four cell lines.

Compound	KB	MCF-7	Hep G2	K562
1	–[a]	–	–	–
2	–	–	–	–
3	–	21.99	–	4.59
4	–	–	–	–
Doxorubicin hydrochloride[b]	0.116	0.78	0.76	0.68

Notes: [a] IC_{50} value > 30 μg/ml.
[b] Used as a positive control.

191.3). However, H-6 showed no NOE correlations with H-5 and H-7, indicating that they were geometrically well apart. Thus, the α-, β-, α-, and α-orientations were proposed for H-5, H-6, H-7, and H-8, respectively.

Cochlione C (**3**) shared a molecular formula of $C_{11}H_{14}O_5$ with compounds **1** and **2** as deduced from the HR-ESI-MS spectrum ($[M + Na]^+$ at *m/z* 249.0733). The ^1H and ^{13}C NMR, ^1H–^1H COSY, and HMBC spectra of **3** were very similar to those of **1**, suggesting that the planar structure was identical to that of **1**. The given relative configuration of **3** was obtained through the analysis of the NOESY spectrum, and H-5, H-6, H-7, and H-8 were evidenced to be α-, α-, β-, and α-oriented by the NOE correlations of H-6 (δ 3.75) with H-5 (δ 3.45) and H-8 (δ 4.45).

Cochlione D (**4**) had a molecular formula of $C_{11}H_{16}O_6$ as demonstrated by its HR-ESI-MS spectrum ($[M + Na]^+$ at *m/z* 267.0843), 18 amu (H_2O) more and one unsaturation degree less than those of **1**–**3**. The ^1H and ^{13}C NMR spectral data of **4** were similar to those of **1**, while the typical carbon chemical shifts (~55 ppm) of oxirane methine were moved downfield, suggesting that the epoxide might have been hydrolyzed. The gross planar structure was affirmed by HMBC and ^1H–^1H COSY experiments. In the NOESY spectrum, the cross-peaks between proton pairs of H-5/H-7 and H-6/H-8 indicated that H-5, H-6, H-7, and H-8 were α-, β-, α-, and β-oriented.

In the MTT bioassay, the new metabolites **1**–**4** were tested for their inhibitory activity against human nasopharyngeal epidermoid (KB), human breast adenocarcinoma (MCF-7), human hepatoma (Hep G2), and human chronic myeloid leukemia (K562) cell lines. Compound **3** showed pronounced inhibition on the growth of MCF-7 and K562 cells with IC_{50} values of 21.99 and 4.59 μg/ml, respectively, whereas others were inactive when evaluated at 30.00 μg/ml (Table 3).

3. Experimental

3.1 General experimental procedures

Melting points were measured using an XT-4 Boetius micromelting point apparatus and are uncorrected. Specific rotations were obtained on a Perkin-Elmer 341 digital polarimeter. Circular dichroism (CD) spectra were obtained on a JASCO J-810 CD spectrometer. UV spectra were recorded on a Hitachi U-3000 spectrophotometer and IR spectra were recorded on a Nexus 870 FT-IR spectrometer. HR-ESI-MS spectra were recorded on a Mariner System 5304 mass spectrometer. NMR data were acquired in $CDCl_3$ on a Bruker DRX500 NMR spectrometer with ^1H and ^{13}C NMR observed at 500 and 125 MHz utilizing the solvent signal ($CDCl_3$, δ_H 7.26, δ_C 77.2) as internal references, and chemical shifts were recorded as δ values. Silica gel (200–300 mesh) for column chromatography was provided by Qingdao Marine Chemical Factory (Qingdao, China). Sephadex LH-20 was purchased from

Pharmacia Biotech (Uppsala, Sweden). HPLC analyses were performed with the column of Allsphere ODS-2.5 μm (250 × 4.6 mm), Hitachi pump L-7100, and UV detector L-7400. All other chemicals used in this study were of analytical grade.

3.2 Plant material and fungal identification

The fungus *Cochliobolus* sp. IFB-E039 was isolated from surface-sterilized fresh shoots of an apparently healthy plant *C. dactylon* that was collected in November 2001 in Nanjing, China. The strain was identified according to the following morphological characters: pseudothecia dark brown to black, globose to subglobose, 210–231 μm in diameter, 334–403 μm high, uniloculate, and filled with pseudoparaphyses and asci. The asci were hyaline, cylindrical to clavate, producing 1–8 ascospores per ascus. Ascospores were hyaline, filiform, coiled tightly with 5–9 septa. Filamentous ascospores were coiled tightly in the ascus. These characteristics indicated that it belonged to the *Cochliobolus* genus, which was reinforced by the sequence of its 18S rDNA that gave a 99% sequence similarity to those accessible at the BLASTN of *Cochliobolus* sp., whose 18S rDNA sequence was deposited as FJ235087 in GenBank. A reference living culture was kept at the Institute of Functional Biomolecules, Nanjing University, China.

3.3 Cultivation

The fresh mycelium grown on PDA medium at 28°C for 5 days was inoculated into 1000 ml Erlenmeyer flasks containing 300 ml Czapek liquid medium. After incubation for 4 days at 28 ± 1°C on a rotary shaker at 150 rpm, 20 ml of the culture liquid was transferred as the seed into 1000 ml flasks, each preloaded with the evenly mingled medium composed of sucrose (30 g), $NaNO_3$ (3.0 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), K_2HPO_4 (1.0 g), KCl (0.5 g), yeast extract (1.0 g), and H_2O added appropriately to a volume of 1000 ml. The fungus then grew for 14 days at 28 ± 1°C with the relative humidity in the range of 60–70%.

3.4 Extraction and isolation

After growing for 2 weeks, the mycelium of *Cochliobolus* sp. (0.5 kg, not completely dried) was removed by filtration with the afforded broth extracted at room temperature with EtOAc (5 × 50 liters). Evaporation of the solvent under reduced pressure provided a brown mass (ca. 15 g), which was chromatographed on a silica gel column (7 × 70 cm, 300 g; 200–300 mesh) eluted with a $CHCl_3$–MeOH mixture (100:0, 1000 ml; 100:1, 1200 ml; 100:2, 1200 ml; 100:4, 1200 ml; 100:8, 1000 ml; 100:16, 1000 ml; MeOH, 1000 ml) to afford seven fractions (Fr-1: 1.2 g, Fr-2: 2.6 g, Fr-3: 2.8 g, Fr-4: 1.3 g, Fr-5: 1.0 g, Fr-6: 0.9 g, and Fr-7: 0.6 g). Fr-3 was chromatographed on a Sephadex LH-20 column (60 × 4.5 cm, 120 g; Amersham, Uppsala, Sweden) with $CHCl_3$–MeOH (1:1, 100 ml) and purified by reverse-phase HPLC with MeOH–H_2O (34:66) on a column of Allsphere ODS-2.5 μm (250 × 4.6 mm) at a flow rate of 2.0 ml/min to afford metabolites **1** (7.9 mg, $t_R = 15.213$ min) and **3** (4.8 mg, $t_R = 19.816$ min). Fr-2 fraction was separated on a silica gel column (60 × 4.5 cm) eluted with $CHCl_3$–EtOAc mixtures [100:2, 1500 ml (75 × 20 ml); 66:44, 500 ml (25 × 20 ml)] to afford 100 fractions, and further purifications of the combined 18–33 and 86–92 sub-fractions afforded **2** (20.1 mg) and **4** (4.9 mg), respectively. Repeated gel filtration of Fr-4 over a Sephadex LH-20 column (2 × 50 cm, 50 g; Amersham) with $CHCl_3$–MeOH (1:1, 300 ml) yielded **5** (156 mg), **6** (54 mg), and **7** (127 mg).

3.4.1 Cochlione A (*1*)

A yellow amorphous powder (mp 176–177°C); $[\alpha]_D^{20}$ + 15.3 (c = 0.008, MeOH);

UV (MeOH) λ_{max} (log ε): 202 (4.36), 274.5 (4.73) nm; IR (KBr) ν_{max}: 3395, 2977, 2932, 1667, 1608, 1373, 1247, 1171, 1087, 876, 780, 577 cm^{-1}; CD (MeOH) $\Delta\varepsilon_{276\,nm}$ −12.7, $\Delta\varepsilon_{308\,nm}$ +0.6, $\Delta\varepsilon_{324\,nm}$ −1.71; ^1H and ^{13}C NMR spectral data are tabulated in the text (Tables 1 and 2); HR-ESI-MS: *m/z* 249.0731 $[M + Na]^+$ (calcd for $C_{11}H_{14}O_5Na$, 249.0739).

3.4.2 Cochlione B (*2*)

A white amorphous powder (mp 220–221°C); $[\alpha]_D^{20}$ −81.8 ($c = 0.015$, MeOH); UV (MeOH) λ_{max} (log ε): 202.5 (3.79), 221 (3.75), 274 (3.83) nm; IR (KBr) ν_{max}: 3454, 2978, 2954, 1752, 1661, 1255, 1215, 1032, 967 cm^{-1}; CD (MeOH) $\Delta\varepsilon_{229\,nm}$ +9.27, $\Delta\varepsilon_{287\,nm}$ −39.5, $\Delta\varepsilon_{307\,nm}$ −25.4, $\Delta\varepsilon_{322\,nm}$ −28.3; ^1H and ^{13}C NMR spectral data are listed in Tables 1 and 2; HR-ESI-MS: *m/z* 249.0730 $[M + Na]^+$ (calcd for $C_{11}H_{14}O_5Na$, 249.0739).

3.4.3 Cochlione C (*3*)

A yellow amorphous powder (mp 158–159°C); $[\alpha]_D^{20}$ + 192.1 ($c = 0.033$, MeOH); UV (MeOH) λ_{max} (log ε): 208.5 (5.01), 296 (3.59) nm; IR (KBr) ν_{max}: 3390, 2975, 2833, 1635, 1585, 1407, 1372, 1253, 1214, 1116, 999, 836 cm^{-1}; CD (MeOH) $\Delta\varepsilon_{255\,nm}$ −11.7, $\Delta\varepsilon_{292\,nm}$ −43.1; ^1H and ^{13}C NMR spectral data are listed in Tables 1 and 2; HR-ESI-MS: *m/z* 249.0733 $[M + Na]^+$ (calcd for $C_{11}H_{14}O_5Na$, 249.0739).

3.4.4 Cochlione D (*4*)

A yellow amorphous powder (mp 191–192°C); $[\alpha]_D^{20}$ + 248.4 ($c = 0.031$, MeOH); UV (MeOH) λ_{max} (log ε): 203 (3.60), 271 (3.76) nm; IR (KBr) ν_{max}: 3359, 2925, 2855, 1662, 1609, 1458, 1373, 1250, 1170, 1092, 1042, 996, 875, 569 cm^{-1}; CD (MeOH) $\Delta\varepsilon_{271\,nm}$ +11.1, $\Delta\varepsilon_{300\,nm}$ +1.53, $\Delta\varepsilon_{329\,nm}$ +5.03; ^1H and ^{13}C NMR spectral data are given in Tables 1 and 2; HR-ESI-MS: *m/z* 267.0843 $[M + Na]^+$ (calcd for $C_{11}H_{16}O_6Na$, 267.0844).

3.5 Cytotoxicity assay

The *in vitro* cytotoxic activity was determined by the MTT colorimetric method [13]. KB, MCF-7, Hep G2, and K562 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (50 μg/ml). The cells were harvested at the log phase of growth, and then seeded into 96-well plates (100 μl/well at a density of 1×10^5 cells/ml). After 24 h incubation at 37°C and 5% CO_2 to allow cell attachment, the cultures were exposed to the tested compounds at various concentrations for 48 h. Then, MTT solution (5.0 mg/ml in PBS) was added (10 μl/well), and the plates were further incubated for 4 h at 37°C and 5% CO_2. Finally, the cell culture supernatant was removed and the formazan crystals formed were dissolved by adding DMSO (150 μl/well). Absorption at 570 nm was measured with an ELISA plate reader, and the IC_{50} value was defined as the concentration at which 50% survival of cells was discerned.

Acknowledgements

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