Cytotoxic Benzo[*j*]fluoranthene Metabolites from *Hypoxylon truncatum* IFB-18, an Endophyte of *Artemisia annua*

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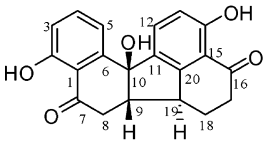
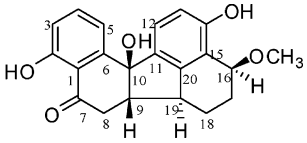
Two new benzo[*j*]fluoranthene-based secondary metabolites named daldinone C (**1**) and daldinone D (**2**), along with two known metabolites, altechromone A and (4*S*)-5,8-dihydroxy-4-methoxy-α-tetralone, were isolated from the $CHCl_3$/MeOH (1:1) extract of a solid culture of the endophyte *Hypoxylon truncatum* IFB-18 harbored inside the symptomless stem tissue of *Artemisia annua*. The structures of the new compounds were elucidated by MS and 1D and 2D NMR spectra and by X-ray diffraction analysis. Their absolute configurations were determined unambiguously by a combination of their CD data and the established exciton chirality rule. Compounds **1** and **2** were substantially cytotoxic against SW1116 cells, with IC_{50} values of 49.5 and 41.0 μM, respectively, comparable to that (37.0 μM) of 5-fluorouracil. The biosynthetic pathway for **1** and **2** was postulated with the natural occurrence of benzo[*j*]fluoranthene analogues discussed in brief.

In our investigations of endophytic fungi for structurally novel and biologically active secondary metabolites,[1,2] *Hypoxylon truncatum* (strain No. IFB-18) (Xylariaceae), an endophytic fungus residing inside symptomless stems of *Artemisia annua*, was shown to be capable of producing cytotoxic substances. Previously, ascomycetes, including *Hypoxylon* species, were reported to produce secondary metabolites such as azaphilones,[3] diterpenes,[4] cytochalasins,[5] and various aromatic compounds,[6,7] some of which exhibit potent antibiotic and antitumor activities.[8] Azaphilones and some other metabolites were demonstrated to be of chemotaxonomic significance in the classification of the *Hypoxylon* species.[9] Here we describe the isolation and characterization of two new cytotoxic benzo[*j*]fluoranthene-based metabolites named daldinone C (**1**) and daldinone D (**2**), as well as the bioactivity and the chemotaxonomic value of these two metabolites. The biosynthetic pathway for **1** and its analogues is also postulated.



1



2

The $CHCl_3$/MeOH (1:1) extract of the fermented product of *H. truncatum* afforded two new benzo[*j*]fluoranthene derivatives named daldinone C (**1**) and daldinone D (**2**), along with the known metabolites altechromone A[10] and (4*S*)-5,8-dihydroxy-4-methoxy-α-tetralone.[11]

Daldinone C (**1**) was obtained as colorless prisms. The molecular fomula $C_{20}H_{16}O_5$ was determined from the quasimolecular ion at *m*/*z* 359.0883 (calcd for $C_{20}H_{16}O_5Na$, 359.0890) in its high-resolution electrospray ionization mass spectrum (HRESIMS) and from its ^1H and ^{13}C NMR data. The ^1H NMR spectrum of **1** showed three methylenes, two methines, five aromatic protons, and two phenolic protons (δ 11.0 and 12.4). The ^{13}C NMR spectrum (Table 1) exhibited well-resolved resonances for 20 carbon atoms including two ketones (δ 203.5 and 203.9), two phenols (δ 161.7 and 162.5), and a tertiary alcohol (δ 80.7). From the $^1H-^1H$ COSY spectrum of **1**, a coupling sequence from H-8 to H-17 could be established.

Table 1. ^1H and ^{13}C NMR Data of **1** ($CDCl_3$) and **2** (pyridine-d_5)

	1		**2**	
position	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	116.1 s		118.5 s	
2	162.5 s		164.0 s	
3	118.4 d	6.89 d (8.2)	118.4 d	6.90 d (7.8)
4	138.3 d	7.50 dd (8.2, 8.0)	139.4 d	7.43[a]
5	120.4 d	7.14 d (8.0)	123.6 d	7.58 d (7.5)
6	143.8 s		148.8 s	
7	203.5 s		207.0 s	
8α	36.5 t	2.92 dd (17.5, 1.5)	38.8 t	2.88 dd (17.5, 1.3)
8*β*		3.29 dd (17.5, 5.7)		3.36 dd (17.5, 5.6)
9	56.6 d	2.64 ddd (10.3, 5.7, 1.5)	58.5 d	2.69 ddd (10.9, 5.6, 1.3)
10	80.7 s		81.3 s	
11	149.6 s		140.8 s	
12	131.9 d	7.81 d (8.1)	126.4 d	7.77 d (8.0)
13	117.6 d	6.91 d (8.1)	117.4 d	7.04 d (8.0)
14	161.7 s		159.6 s	
15	115.0 s		122.2 s	
16	203.9 s		73.5 d	4.59 brs
17α	38.7 t	2.57 ddd (17.8, 13.7, 4.6)	30.1 t	1.30 brdd (14.1, 13.9)
17*β*		2.72 ddd (17.8, 3.8, 2.9)		2.16 brd (14.2)
18α	29.0 t	2.42 dddd (17.2, 4.6, 4.5, 2.9)	24.6 t	1.72 m
18*β*		1.81 dddd (17.2, 13.7, 11.2, 3.8)		1.47 brddd (16.7, 13.9, 11.2)
19	42.4 d	2.79 ddd (11.2, 10.3, 4.5)	45.6 d	2.37 ddd (11.2, 10.9, 4.7)
20	137.6 s		145.9 s	
HO-2		12.4 s		12.9 s
HO-14		11.0 s		11.3 s
CH_3O-16			59.3 q	3.44 s

[a] Partially overlapped with the solvent residual proton peak at δ 7.44.

The HMBC correlations of OH-2 to C-2 and C-3 and of HO-14 to C-13, C-14, and C-15 placed hydrogen-bonded hydroxyls at C-2 and C-14, respectively. HMBC correlations from C-10 to H-5, H-8, H-9, and H-19 located the tertiary hydroxyl at C-10. These observations showed that **1** possessed the same planar structure as daldinone A.[12] However, in the case of daldinone A,[12] an NOE effect was detected between H-9 and H-19, whereas none between the two protons was discerned in the case of **1**. This observation and their distinction in physical data suggested that **1** and daldinone

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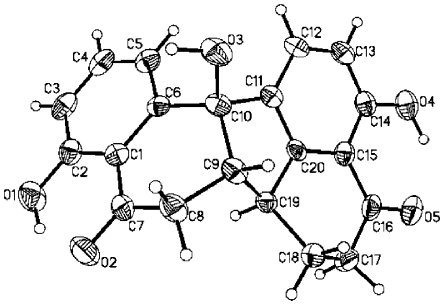
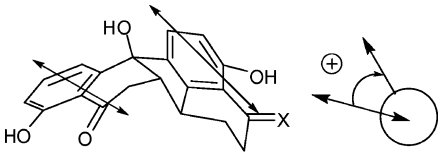


Figure 1. ORTEP drawing of **1**.



1 X = O
2 X = α-H, β-OCH_3

Figure 2. Positive (+) torsion angle between two chromophores of **1** and **2**.

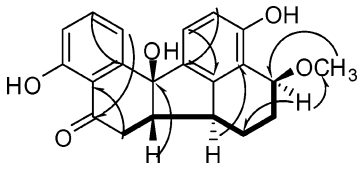


Figure 3. Important $^1H-^1H$ COSY correlations (bold lines) and HMBC correlations (arrows) of **2**.

A should be different compounds and thus gave us an impetus to scrutinize its configuration. Compound **1** was then subjected to an X-ray diffraction analysis, which indicated that the dehedral angle between H-9 and H-19 was 163.2° and that H-9 was *trans* to H-19 and *cis* to the 10-hydroxy group (Figure 1). With this reassigned relative configuration of **1** in hand, the absolute stereochemistry of the metabolite was also re-established on the basis of its CD spectrum and exciton coupling analysis. Thus, a pair of typical exciton-split Cotton effects at 209.3 ($\Delta\epsilon = -27.7$) and 229.3 ($\Delta\epsilon = +44.7$) nm in its CD spectrum highlighted the positive (+) torsion angle between the two tetralone chromophores of the molecule according to the exciton chirality rule (Figure 2).[13] Accordingly, the absolute configurations of C-9, C-10, and C-19 of daldinone C (**1**) were deduced to be *S*, *R*, and *S*, respectively.

The new metabolite **2**, named daldinone D, was obtained as a whitish powder. Its molecular fomula $C_{21}H_{20}O_5$ was disclosed by its HRESIMS ($[M + Na]^+$: 375.1198, calcd for $C_{21}H_{20}O_5Na$, 375.1203) and ^1H and ^{13}C NMR data. The ^1H NMR spectrum of **2** exhibited a methoxy group (δ 3.44), three methylenes, three methines (an oxygenated one resonating at δ 4.59), five aromatic protons, and two hydroxyls (δ 11.3 and 12.9). The ^1H and ^{13}C NMR data of **2** were very similar to those of **1**, suggesting that they were congeners (Table 1). However, one of the two ketone groups in **1** was replaced by a methoxylated methine in **2**, which was confirmed by the $^1H-^1H$ COSY spectrum of **2**, indicating the coupling sequence from the C-8 methylene through the C-16 methine (Figure 3). The 16-methoxy group was evidenced from the HMBC correlation of C-16 with the three-proton singlet at δ 3.44, showing as well NOE cross-peaks with the H-16 singlet (δ 4.59) and the H-17β doublet at δ 2.16 (Figure 4).

The relative configuration of **2** was evidenced from its NOESY data. The H-17α signal at δ 1.30 had clear NOE correlations with H-16 (δ 4.59) and H-19 (δ 2.37) that did not correlate with H-9 at δ 2.69 (Figure 4). Concerning its absolute stereochemistry, Cotton effects at 220.8 ($\Delta\epsilon = -44.2$) and 236.6 ($\Delta\epsilon = +32.2$) nm in the

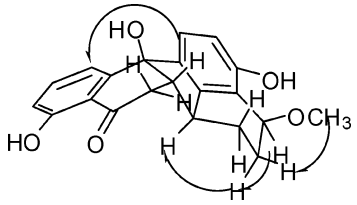


Figure 4. Key NOE correlations for **2**.

CD spectrum of **2** illustrated again a positive (+) torsion angle between its two chromophores, as discerned with **1** (Figure 2).[13] Thus, compound **2** possessed 9*S*-, 10*R*-, 16*S*-, and 19*S*-configurations.

Compound **2** was structurally too close to that of **1** to forget the possibility that the former could be an artifact of the latter formed during the fractionation procedure using MeOH. However, the formation of **2** from **1** necessitates the reduction and methoxylation of the C-16 ketone group of **1**, which could not be accomplished in the enzyme-free isolation process. This reaction-based rationalization discarded the generation of **2** from **1** during isolation.

Compounds **1** and **2** were cytotoxic to the SW1116 cell line, with IC_{50} values of 49.5 and 41.0 μM, respectively, close to that (37.0 μM) of 5-fluorouracil co-tested as a positive control. However, no cytotoxicity was observed with the other two metabolites.

This work describes for the first time the production of cytotoxic benzo[*j*]fluoranthene-based daldinones C (**1**) and D (**2**) by the endophytic *H. truncatum*. Biosynthetically, the benzo[*j*]fluoranthene framework they shared was probably derived from [1,1′-binaphthalene]-4,4′,5,5′-tetrol (BNT) of pentaketide origin[14] as proposed for truncatone.[15] Thus, metabolites **1** and **2** could be generated from BNT after experiencing conjugate addition, rearrangement, oxidation, and reduction as formulated in Scheme 1. Specifically, daldinone A failed to be detected in the endophyte culture, highlighting that it could not produce an intermediate-epimerization isomerase that could have occurred in the culture of other fungal strains such as *Daldinia concentrica*[12] (Scheme 1).

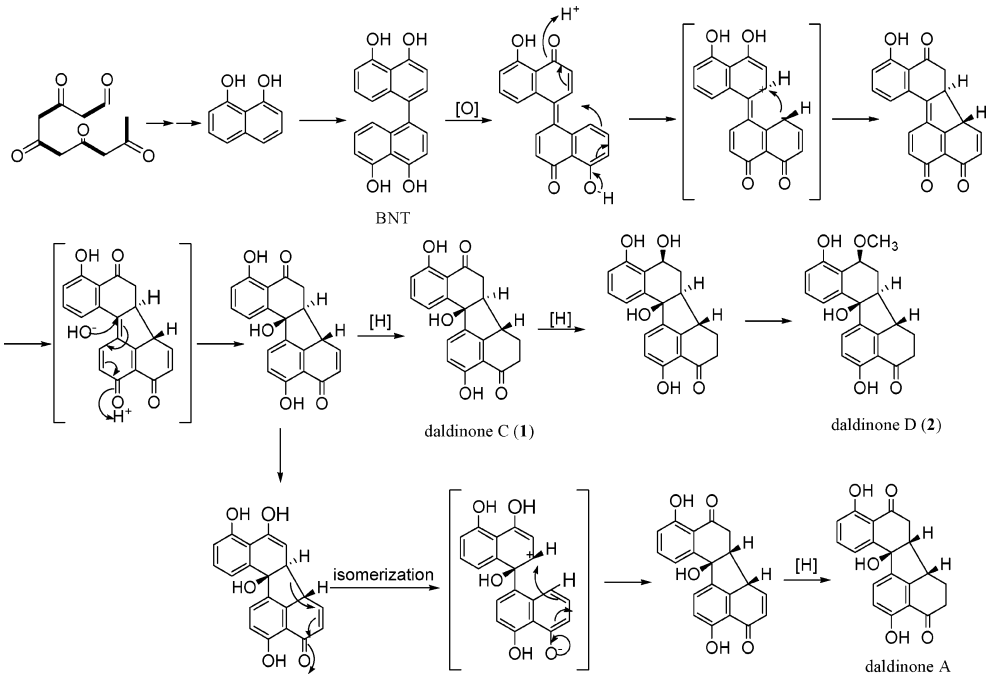
Twelve naturally occurring benzo[*j*]fluoranthene-based metabolites have been reported, all produced by fungi belonging to the genera *Hypoxylon*,[15,16] *Daldinia*,[12] *Bulgaria*,[17] *Clardosporium*,[18] and *Hortaea*.[19] Interestingly, no metabolite with a benzo[*j*]fluoranthene framework has been characterized as a phytochemical, although some plants such as *Juglans* species (Juglandaceae) contain tetralone-based constituents that could also derive from a pentaketide.[11,20] Therefore, benzo[*j*]fluoranthene-based metabolites such as **1** and **2** could be helpful in the classification of morphologically similar or undifferentiated species of these fungal genera.

Experimental Section

General Experimental Procedures. Melting points were measured on an XT-4 apparatus and were uncorrected. Optical rotations were measured on a WXG-4 polarimeter. The UV spectra were acquired on a Hitachi U-3000 spectrophotometer and CD spectra on a Jasco J-810 circular dichroism spectrometer. IR spectra were measured on a Nexus 870 FT-IR spectrometer. ^1H and ^{13}C NMR, HMQC, HMBC, and NOESY spectra were accomplished on Bruker DPX-300 and DRX-500 NMR spectrometers using TMS as internal standard. The HRESIMS was recorded on a Mariner mass spectrometer. Silica gel (200−300 mesh) for column chromatography and silica GF_{254} (10−20 μm) for TLC were products of Qingdao Marine Chemical Factory, China, and Sephadex LH-20 was from Pharmacia Biotech, Sweden. HPLC was accomplished on a Hitachi L-7110 pump equipped with an Apollo C18 5 μm column (250 × 4.6 mm) from Alltech Associates, Inc.

Fungal Material. The strain of *H. truncatum* IFB-18 was isolated from the surface-sterilized fresh stems of *Artemisia annua* collected in early July 2003 in the suburb of Nanjing, China. The specimen of *A. annua*, authenticated by Prof. L. X. Zhang, was preserved under the number YC-03-07-06 in the Herbarium of Nanjing University. The fungal strain was identified by comparing its morphological charac-

Scheme 1. Possible Biosynthetic Pathway for **1**, **2**, and Daldinone A



teristics[21] and 18S rDNA sequence with those standard records. The experimental data and observations led to the identification of the strain as *H. truncatum*, which was deposited under the identification number IFB-E0018 at the Institute of Functional Biomolecules, Nanjing 210093 (China).

The fresh mycelium of the endophyte strain IFB-18 grown on PDA agar plates for 7 days was inoculated into 1000 mL flasks containing 400 mL of PD medium. After 4-day incubation at 25 °C on a rotary shaker at 140 rpm, the liquid culture (10 mL for each flask) was transferred into 400 flasks of solid millet medium (7.5 g of millet, 7.5 g of bran, 0.5 g of yeast extract, 0.1 g of sodium tartrate, 0.1 g of sodium glutamine, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.1 mL of corn oil, and 30 mL of H_2O) and then cultured at 25 °C for 30 days. At the end of cultivation the culture was harvested, dried, and powdered.

Extraction and Isolation. The fermentation product was extracted three times with a $CHCl_3$/MeOH (1:1, v/v) mixture (3 × 4 L). In vacuo evaporation of the solvent yielded an oily residue (440 g). Then 500 mL of MeOH was added to the residue and the mixture was refluxed at 80 °C until the residue was fully dissolved. After being cooled to room temperature, the solution was kept at −20 °C overnight to precipitate the waxy substrate. After removing the precipitate by filtration, the filtrate was concentrated in vacuo to give a brown residue (270 g). The residue was then divided into nine fractions by column chromatography on silica gel eluted by a gradient of $CHCl_3$/MeOH from 1:0 to 0:1 (v/v). Fraction 2 (11.8 g) was rechromatographed on silica gel (gradient of $CHCl_3$/MeOH from 1:0 to 1:1 (v/v)) to give five fractions. Fraction 2-2 was recrystallized in MeOH to afford compound **1** (210 mg). Fraction 3 (8.6 g) was chromatographed on silica gel (gradient of $CHCl_3$/MeOH from 0:1 to 1:1, v/v) to give six fractions. Fraction 3-2 was subject to gel filtration on Sephadex LH-20 ($CHCl_3$/MeOH, 1:1) and then purified by preparative HPLC (MeOH/H_2O, 65:35) to afford compound **2** (2 mg). Fraction 3-3 was also subjected to gel filtration on Sephadex LH-20 followed by preparative HPLC (MeOH/H_2O, 60:40) to give altechromone A (6 mg) and (4*S*)-5,8-dihydroxy-4-methoxy-α-tetralone (2.5 mg).

Daldinone C (1): colorless prisms; mp 210−211 °C (dec), $[\alpha]^{20}_D$ −295.1 (*c* 0.8, MeOH); UV (EtOH) λ_{max} (log ϵ) 224 (4.53), 259 (4.25), 337 (3.92) nm; CD (EtOH) λ_{max} nm ($\Delta\epsilon$) 209.3 (−27.7), 229.3 (+44.7), 255.1 (−28.5); IR (KBr) ν_{max} 3396.3, 1637.1, 1451.0, 1355.8, 1325.9, 1035.3 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 359.0883 ([M + Na]$^+$, calcd for $C_{20}H_{16}O_5Na$, 359.0890).

X-ray Crystallographic Analysis of 1. A colorless prism of **1** (0.24 × 0.22 × 0.22 mm) was selected for data collection, and its structure was solved by the direct method using SHELXL-97.[22] Crystal data of compound **1**: $C_{20}H_{16}O_5$, M_r = 336.33, orthorhombic, space group *P*2(1)2(1)2(1), a = 16.0578(10) Å, b = 16.7741(10) Å, c = 22.9457(13) Å, $\alpha = \beta = \gamma = 90°$, V = 6180.5(6) Å^3, Z = 16, $F(000)$ = 2816, λ(Mo Kα) = 0.71073 Å, μ(Mo Kα) = 0.090 cm^{-1}, D_x = 1.446 g·cm^{-1}, T_{min}/T_{max} = 0.97/0.98, reflections/parameters = 8259/901, goodness of fit on F^2 = 1.003, R_1, wR_2 [$I \geq 2\sigma(I)$] = 0.0620/0.1254, R_1, wR_2 (all data) = 0.0864/0.1316. The structure of **1** was refined by SHELXL-97. All non-hydrogen atoms were refined anisotropically. All hydrogen atoms calculated on ideal positions were refined with isotropic thermal parameters and included in the calculations of the structure factors.

Daldinone D (2): white, amorphous powder; $[\alpha]^{20}_D$ −223.6 (*c* 0.06, MeOH); UV (EtOH) λ_{max} (log ϵ) 209 (4.32), 259 (3.90), 291 (3.65), 343 (3.64) nm; CD (EtOH) λ_{max} ($\Delta\epsilon$) 220.8 (−44.2), 236.6 (+32.2), 256.7 (−10.1); IR (KBr) ν_{max} 3390.8, 2924.6, 1639.5, 1452.0, 1384.3, 1041.1 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 375.1198 ([M + Na]$^+$ cacld for $C_{21}H_{20}O_5Na$, 375.1203).

Cytotoxic Assay. Cytotoxic activity was determined in vitro via the MTT colometric method.[23] SW1116 cells were grown in RPMI medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (50 μg/mL). Cells were harvested at the log phase of growth and seeded in 96-well plates (100 μL/well at a density of 2 × 10^5 cells/mL). After 24 h incubation at 37 °C and 5% CO_2 to allow cell attachment, cultures were exposed to various concentrations of the isolated compounds for 48 h. Finally, MTT solution (2.5 mg/mL in PBS) was added (40 μL/well). Plates were further incubated for 4 h at 37 °C, and the formazan crystals formed were dissolved by adding 150 μL/well of DMSO. 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.

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Supporting Information Available: This material, ^1H, ^{13}C, NOESY, and CD spectra of **1** and **2**, is available free of charge via the Internet at http://pubs.acs.org.

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(22) Crystallographic data for the structure reported in this paper have been deposited in the Cambridge Crystallographic Data Centre, as CCDC No. 615759. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).
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