

A New Diphenyl Ether from Marine-derived Fungus *Aspergillus* sp. B-F-2

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Abstract A new diphenyl ether dimethyl 2,3'-dimethoxysoate (**1**) together with three known compounds monomethylsulochrin (**2**), emodin (**3**), and questin (**4**) were isolated through bioassay-guided fractionations from the culture of a marine-derived fungus *Aspergillus* sp. B-F-2. The structures of these compounds were determined by spectroscopic methods. Cytotoxicities of compounds **1** and **2** against K562 cell line were preliminarily evaluated by the MTT method and flow cytometry.

Keywords *Aspergillus* sp., diphenyl ether, asterric acid, anthraquinone, cytotoxicity

Introduction

In our research for new anticancer microbial metabolites, a culture broth of a marine-derived fungus *Aspergillus* sp. B-F-2, exerted significant cytotoxic activity against K562 cell line. Bioassay-guided isolation led to one new asterric acid derivative dimethyl 2,3'-dimethoxysoate (**1**), and three known fungal metabolites monomethylsulochrin (**2**), emodin (**3**), and questin (**4**) (Fig. 1).

Asterric acid, possessing a diphenyl ether structure, was the first non-peptide endothelin (ET) binding inhibitor discovered [1, 2]. In this paper we mainly report the isolation and structure determination of the new diphenyl ether (**1**), and its preliminary *in vitro* cytotoxicity against the

K562 cell line. These kinds of compounds are formed biosynthetically from the anthraquinone emodin, *via* sulochrin, and grisandienes such as geodin was previously reported [3, 4].

Materials and Methods

Fungal Material

The fungus *Aspergillus* sp. B-F-2 was isolated from the marine sediments collected in Behai Bay, China. It was preserved in the China Center for Type Culture Collection (patent depositary number: B-F-2 CCTCC M 205148). Working stocks were prepared on Potato Dextrose agar slants stored at 4°C.

Fermentation and Extraction

The fungus was grown under static conditions at 24°C for 25 days in 1000 ml×150 conical flasks containing the liquid medium (300 ml/flask) composed of glucose (20 g/liter), peptone (5.0 g/liter), malt extract (3.0 g/liter) and yeast extract (3.0 g/liter) and sea-water after adjusting its pH to 7.0. The fermented whole broth (45 liter) was

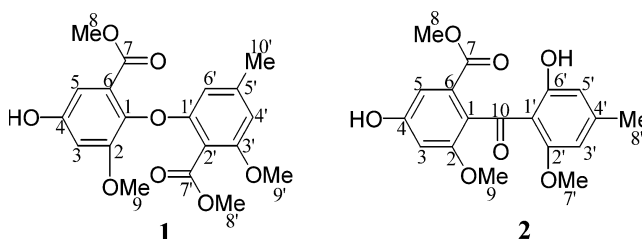


Fig. 1 Structures of compounds **1** and **2**.

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filtered through cheese cloth to separate the supernatant from the mycelia. The former was concentrated under reduced pressure to about a quarter of the original volume and then extracted three times with ethyl acetate to give an ethyl acetate solution, while the latter was extracted three times with acetone. The acetone solution was concentrated under reduced pressure to afford an aqueous solution. The aqueous solution was extracted three times with ethyl acetate to give another ethyl acetate solution. Both ethyl acetate solutions were combined and concentrated under reduced pressure to give a crude extract (22.0 g).

Purification

The crude extract (22.0 g) showed strong cytotoxicity against K562 cells at 50 $\mu\text{g/ml}$. It was separated into 12 fractions on a silica gel column using a step gradient elution of CHCl_3 :MeOH. The bioactive fraction 3, eluted with CHCl_3 :MeOH 98:2 (2.9 g), was further separated on another silica gel column using a step gradient elution of petroleum ether:acetone to give compounds **3** (100 mg) and **4** (200 mg). The bioactive fraction 5, eluted with CHCl_3 :MeOH 95:5 (1.6 g), was subjected to a Sephadex LH20 column chromatography (100% MeOH) and further purified by semipreparative HPLC [YMC-Pack ODSA, 10 \times 250 mm, 5 μm , 4 ml/minute] eluted with MeOH:H₂O (60:40, 4.0 ml/minute) to give compounds **1** (30 mg) and **2** (58 mg).

Biological Assays

The human chronic myelogenous leukemia cell line K562 was grown in RPMI 1640 culture medium supplemented with 10% fetal calf serum, 100 U/ml penicillin G, and 100 $\mu\text{g/ml}$ streptomycin at 37°C in 5% CO₂/100% humidified air.

Cytotoxic activity was evaluated by the MTT method [5] using the K562 cell line. 200 μl of the cell suspensions at a density of 5 \times 10³ cell/ml were plated in 96 well microtiter plates under the above conditions. Then 2 μl of the test compound solutions (in DMSO) at different concentrations was added to each well and further incubated for 72 hours under the same conditions. 20 μl of the MTT solution (5 mg/ml in PBS) was added to each well and incubated for 4 hours. One hundred fifty μl of the spent medium containing MTT was then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a SPECTRA MAX PLUS plate reader at 570 nm.

For apoptosis assessment, cells were plated in 12-well cell culture plates (5 \times 10⁴ cells/well) in triplicate and treated with DMSO (control) or compounds (**1** and **2**) at the indicated concentrations (Table 2) for 72 hours. Cells were

Table 1 600 MHz ¹H and 150 MHz ¹³C NMR data of compound **1** in CDCl₃

Position	1	
	δ_{C}	δ_{H} (J in Hz)
1	136.2 s	—
2	154.2 s	—
3	105.1 d	6.69 (1H, d, 2.9)
4	153.5 s	—
5	108.5 d	7.00 (1H, d, 2.9)
6	125.9 s	—
7	166.3 s	—
8	52.5 q	3.74 (3H, s)
9	56.4 q	3.72 (3H, s)
1'	156.5 s	—
2'	109.9 s	—
3'	157.3 s	—
4'	105.0 d	6.36 (1H, br s)
5'	141.3 s	—
6'	106.4 d	5.86 (1H, br s)
7'	167.0 s	—
8'	52.4 q	3.91 (3H, s)
9'	55.9 q	3.82 (3H, s)
10'	22.1 q	2.19 (3H, s)
4-OH		5.97 (1H, s)

harvested, washed twice with PBS and the cellular DNA was stained with 200 μl of propidium iodide (PI, 50 $\mu\text{g/ml}$, RNase 1 $\mu\text{g/ml}$, Triton X-100 0.1%). After incubation at 4°C for 20 minutes, cells were analyzed on a flow cytometer.

Results and Discussion

Physico-chemical Properties

Compound **1**: white needles; mp 159~161°C; HRESIMS m/z : 375.1094 [M-H]⁻; (calcd for C₁₉H₁₉O₈, 375.1080); UV λ_{max} (CHCl₃) nm (log ϵ) 255 (2.30), 308 (2.35); IR ν_{max} (KBr) cm⁻¹ 3377, 2956, 1707, 1611, 1588, 1453, 1314, 1281, 1099, 1061, 1008, 807; ¹H and ¹³C NMR (see Table 1).

Compound **2**: white needles; mp 199~200°C; ESIMS m/z : 369 [M+Na]⁺; UV λ_{max} (CHCl₃) nm (log ϵ) 260 (2.09), 286 (2.27); IR ν_{max} (KBr) cm⁻¹ 3403, 2992, 1692, 1606, 1447, 1354, 1215, 1062, 817, 638; ¹³C NMR (acetone-*d*₆, 150 MHz): δ_{C} =128.2 (s, C-1), 158.7 (s, C-2), 103.7 (d, C-3), 158.0 (s, C-4), 108.1 (d, C-5), 129.4 (s, C-6), 166.7 (s, C-7), 52.2 (q, C-8), 56.4 (q, C-9), 200.4 (s, C-

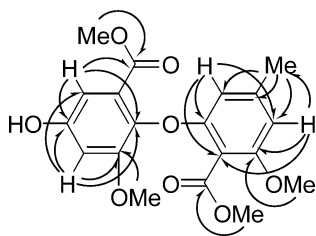


Fig. 2 HMBC analysis of compound **1**.

10), 111.4 (s, C-1'), 162.1 (s, C-2'), 103.9 (d, C-3'), 148.5 (s, C-4'), 111.1 (d, C-5'), 164.9 (s, C-6'), 56.2 (q, C-7'), 22.2 (q, C-8'); ^1H NMR (acetone- d_6 , 600 MHz): δ_{H} =6.74 (1H, d, J =2.2, H-3), 7.02 (1H, d, J =2.2, H-5), 3.64 (3H, s, H-8), 3.69 (3H, s, H-9), 6.24 (1H, br s, H-3'), 6.37 (1H, br s, H-5'), 3.40 (3H, s, H-7'), 2.27 (3H, s, H-8').

Structure Determination

The bioactive ethyl acetate extract of the *Aspergillus* sp. was chromatographed on silica gel columns and reversed-phase preparative HPLC to give four pure compounds (**1**~**4**). Compounds **2**~**4** were identified as monomethylsulochrin (**2**) [6, 7], emodin (**3**), and questin (**4**) respectively, by comparison of their spectroscopic data with those reported in the literature.

The molecular formula of compound **1** was determined to be $\text{C}_{19}\text{H}_{20}\text{O}_8$ from the negative HRESIMS spectrum (m/z 375.1094, calcd for $[\text{M}-\text{H}]^-$ 375.1080). The UV absorption maxima at 255 and 308 nm in CHCl_3 resembled those of asterric acid derivatives, which possess the biphenyl ether structure [8]. In the IR spectrum, an absorption band at 1707 in combination with the broad band at 3377 cm^{-1} suggested the presence of an ester and a hydroxyl group, respectively. In the ^1H -NMR spectrum, one methyl singlet at δ 2.19, four methoxyl singlets at δ 3.72, 3.74, 3.82, 3.91, four benzen hydrogen signals at δ 5.86, 6.36, 6.69, 7.00, and one exchangeable hydrogen proton signal at δ 5.97 were observed. The ^{13}C NMR showed a total of 19 resonances. After the assignment of the carbons bearing hydrogens by the HMQC experiment, the structure of two aromatic ring moieties were determined by the HMBC experiments shown in Fig. 2. The two aromatic rings were bonded by an oxygen through an analysis of the carbon signals at C-1 and C-1', combined with the molecular formular. On the basis of the above data, the structure of **1** was determined.

Cytotoxicities and Apoptosis

The quantification of cellular growth, including viability, apoptosis and different phases of the cell cycle, has become an essential parameter in the evaluation of anticancer

Table 2 Distribution of the K562 cells within cell cycle and apoptosis^a

Compound	Concentration (μM)	Distribution of the cells (%)			
		Apo	G ₀ /G ₁	S	G ₂ /M
Control	—	0.7	50.2	38.3	12.7
1	100	11.5	29.0	56.4	4.6
	10	0.7	47.6	41.2	11.2
	1	0.8	54.2	33.2	12.6
	0.1	0.7	51.4	34.1	14.3
	0.01	0.3	51.8	39.0	10.3
2	100	0.5	49.8	37.3	12.9
	10	0.9	50.6	36.6	12.8
	1	0.5	52.7	37.0	10.3
	0.1	0.2	48.2	37.7	14.1
	0.01	0.8	51.0	34.7	14.1

^a Data were obtained from the histograms. Numbers in the column Apo represent relative percentage of the apoptotic cells counted in the sub-G₀/G₁ peak region to the total cell numbers. The proportion of cells in each phase of the cell cycle (G₀/G₁, S, and G₂/M) was analyzed with the computer software CellQuest.

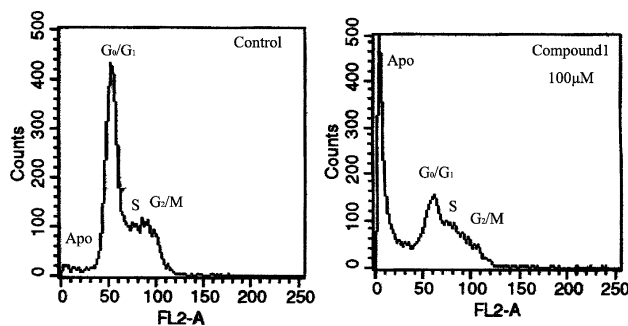


Fig. 3 Effects of **1** on K562 cell cycle and apoptosis.

The given result is a representative of three independent experiments.

activities of compounds. To evaluate the potential anticancer effect of compounds **1** and **2**, cell viability and cellular DNA analyses were performed using K562 cells. A decrease in reduced MTT concentrations is generally observed in the presence of compound **1** (IC_{50} =76.5 μM) whereas compound **2** has little effect (IC_{50} >100 μM).

In the cell cycle and apoptosis assay, as shown in Table 2, **1** at 100 μM increased the percentage of cells in the S phase of the cell cycle from 38.3% (Control) to 56.4%. Meanwhile, after 72 hours treatment with **1** at the same concentration, the percentage of apoptotic cells was also increased (11.5%) as compared with control group (0.7%) (Fig. 3). In contrast, **2** at each concentration had

little effect on cellular DNA, as compared with the control group. Collectively, we demonstrate for the first time that **1** inhibits cell viability through S-phase blockage of the cell cycle and cell apoptosis.

Compounds, possessing diphenyl ether skeleton, are common constituents of fungi metabolites, such as *Pestalotiopsis* sp. [8], *Aspergillus* sp. [2], and *etc.* Their reported bioactivities mainly focus on the endothelin (ET) binding inhibitory activities [1, 8], with nearly no reports on cytotoxicity. Our study demonstrated for the first time that compound having diphenyl ether skeleton showed S phase inhibitory activity against K562 cell line. Therefore it is important to further evaluate the cytotoxicity of diphenyl ether compounds for finding their structure-activity relationships.

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