

Dichlorodiaportinol A – A new chlorine-containing isocoumarin from an endophytic fungus *Trichoderma* sp. 09 from *Myoporum bontioides* A. Gray and its cytotoxic activity

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ABSTRACT

Background: *Myoporum bontioides* A. Gray, an evergreen shrub from the Myoporaceae family, is a commonly used medicinal plant. Many studies have been conducted on the biologically active constituents of whole parts of *M. bontioides*. However, the endophytes of *M. bontioides* have not been intensively investigated. A new chlorine-containing isocoumarin, named dichlorodiaportinol A (1) was isolated from the endophytic fungus *Trichoderma* sp. 09 isolated from the root of *M. bontioides*. Its cytotoxic activity against human breast cancer (MCF-7) and human liver cancer (HepG2) cell lines was evaluated. **Materials and Methods:** Different open silica gel column chromatographic techniques with different solvent systems were used for the separation of the constituents of the ethyl acetate extract of the culture broth of the endophytic fungus *Trichoderma* sp. 09. The structure of compound one was identified by analysis of spectroscopic data [one-dimensional (1D), two-dimensional (2D)-nuclear magnetic resonance (NMR), ultraviolet (UV), infrared (IR) and Mass spectrometry (MS)]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay method was used for the evaluation of cytotoxic activity of compound one against MCF-7 and HepG2 cell lines. **Results:** Compound one was identified as 3-(3,3-dichloro-2,3-dihydroxy-propyl)-8-hydroxy-6-methoxy-isochromen-1-one. It inhibited MCF-7 and HepG2 cell lines, with half maximal inhibitory concentration (IC₅₀) values of 17.8 and 39.6 µg/mL, respectively. **Conclusions:** Compound one is a new chlorine-containing isocoumarin with moderate cytotoxic activity against MCF-7 and HepG2 cell lines. Thus, endophytes of *M. bontioides* are worthy of consideration for the development and research of antitumor agents.

Key words: Cytotoxic activity, dichlorodiaportinol A, isocoumarin, *Trichoderma* sp.

INTRODUCTION

Endophytes are microorganisms that reside in the tissues of living plants without causing any damage to the host.^[1] They have proven to be a rich source of structurally unique and biologically active secondary metabolites which are of interest for specific medicinal or agrochemical value.^[2] *Myoporum bontioides* A. Gray belonging to genus *Myoporum*, family Myoporaceae, is a small evergreen shrub distributed along the coastal

regions of southern Kyushu islands through Okinawa, Taiwan, southern China, and Indochina. In China, the decoction of *Myoporum bontioides* A. Gray is usually used as a folk medicine for antidermatosis and as antipyretic and antipsychotic agent.^[3,4] The phytochemistry and pharmacological studies conducted on this medicinal plant have successfully isolated a serial of natural products including sesquiterpenoids, iridoids, monoterpenes, phenylethanoids, and flavonoids etc.^[5-8] However, the chemical and medicinal values of its endophytes seldom have been investigated.

During our continuing search for structurally new bioactive secondary metabolites from endophytes from *M. bontioides*,^[9] we examined the chemical constituents of

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Trichoderma sp. 09, an endophytic fungus isolated from the root of the semi-mangrove plant. In the study, we describe the isolation and characterization of a new chlorine-containing isocoumarin, 3-(3,3-dichloro-2,3-dihydroxy-propyl)-8-hydroxy-6-methoxy-isochromen-1-one, Dichlorodiaportinol A (1) obtained from the culture broth of this fungus. Its *in vitro* cytotoxic activity against human breast cancer (MCF-7) and human liver cancer (HepG2) cell lines were evaluated.

MATERIALS AND METHODS

General

Melting points were detected on X-4 micromelting point apparatus (Cany Precision Instruments Co., Ltd., Shanghai, China), uncorrected. Ultraviolet (UV) absorptions were measured in MeOH on a Shimadzu UV-2450 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Infrared (IR) spectra were obtained on a Nicolet 5DX-Fourier transform infrared (FTIR) spectrophotometer (Thermo Electron Corporation, Madison, USA). Nuclear Magnetic Resonance (NMR) data were recorded on a Bruker AVIII 600MHz NMR spectrometer (Bruker BioSpin GmbH company, Rheinstetten, Germany), using deuterated acetone (CD_3COCD_3) as solvent and tetramethylsilane (TMS) as internal standard, and coupling constants (J) are in Hz. Electrospray ionization mass (ESIMS) and high resolution electrospray ionization mass (HRESIMS) were operated on LCQ-DECA-XP (Thermo, USA), and LCMS-IT-TOF (Shimadzu, Japan) mass spectrometers, respectively. Chromatography was carried out on silica gel column (200-300 mesh; Qingdao haiyang chemicals Co., Ltd., Qingdao, China). All other reagents used were analytical grade.

Fungus and cell material

The *Trichoderma* sp. 09 strain was isolated from the root of *M. bontioides* A. Gray collected in Leizhou Peninsula, Guangdong Province, China. Stock cultures were maintained on slant solid cornmeal seawater agar. Plugs of agar supporting mycelia growth were cut and transferred aseptically to a 250 mL Erlenmeyer flask containing 100 mL of liquid medium (glucose 10g/L, Peptone 2 g/L, yeast extract 1 g/L, NaCl 30 g/L). The flask was incubated at 30°C on a rotary shaker for 5–7 days. The mycelium was aseptically transferred to 500 mL Erlenmeyer flasks containing culture liquid (200 mL). The flasks were then incubated at 30°C for 25 days.

Breast MCF-7 and Liver HepG2 cell lines were purchased from the American Type Culture Collection (ATCC). They were cultured in Dulbecco's modification Eagle's medium (DMEM, Invitrogen,

Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 2 mM L-glutamine, 100 µg/mL streptomycin, and 100 U/mL penicillin (Invitrogen). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 . Microplate reader (TECAN, Inc.). Flat-bottom microtiter plates, 96 well were from Falcon, NJ, USA. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for viability assay was purchased from Sigma (St. Louis, Mo., USA).

Extraction and isolation

The cultures (30 L) were filtered through cheesecloth. The filtrate was concentrated to 5 L *in vacuo* below 50°C and extracted five times by shaking with an equal volume of ethyl acetate. The combined extract (18.6 g) was subjected to silica gel column chromatography and eluted with petroleum ether–ethyl acetate (90:10, 80:20, 70:30, 50:50, and 0:100) to afford fractions 1-5 (0.9, 1.6, 2.2, 3.7, and 5.8 g, respectively). Fraction four (3.7 g) was subjected to silica gel column chromatography and eluted with petroleum ether–ethyl acetate in a gradient of ethyl acetate (petroleum ether-ethyl acetate, 100:0-50:50). Fifty-two fractions of 30 ml each were collected, combined on the basis of their thin layer chromatography profiles, and concentrated to dryness to give nine major fractions A–I. Repeated slow recrystallization of Fraction H (85 mg, petroleum ether-ethyl acetate 60:40, v/v) at room temperature from petroleum ether-ethyl acetate (65:35, v/v) yielded 3.2 mg white crystalline needle shape solid of Dichlorodiaportinol A (1).

Assessment of Antitumor Activity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) Assay

Cells were harvested during logarithmic growth phase and seeded in 96-well plates at a density of 1×10^4 cells/mL, and cultured at 37°C in a humidified incubator (5% CO_2) for 24 h, followed by exposure to various concentrations of compound one tested for 48 h. Negative control cells were incubated without test samples. Cells treated with epirubicin served as a positive control. Subsequently, 20 µL of MTT (Genview, Houston, TX, USA) solution (5 mg/mL) was added to each well and mixed, the cells were then incubated for an additional 4 h. Culture supernatant was removed and 150 µL of Dimethyl sulfoxide (DMSO) (Sangon Biotech, Shanghai, China) was added to each well to fully dissolve the MTT-formazan crystals. Cell growth inhibition was determined by measuring the absorbance (Abs) at $\lambda = 570$ nm using a microplate reader and calculated according to the following equation: Growth inhibition = $(1 - OD \text{ of treated cells} / OD \text{ of control cells}) \times 100\%$. The half maximal inhibitory concentrations (IC_{50}) were obtained from liner regression analysis of the concentration-response curves plotted for each tested compound.

RESULTS

Dichlorodiaportinol A (1)

White needles, m.p. (CHCl₃) 137–139°C [α]_D²⁵ + 7.5° (*c* 0.04, CH₃COCH₃). UV (MeOH) λ_{\max} (nm): 243, 280, 326. IR (KBr) ν_{\max} (cm⁻¹): 3,421, 2,925, 1,685, 1,637, 1,570, 1,508, 1,241, 1,207, 1,166, 1,047 cm⁻¹. ¹H-nuclear magnetic resonance (NMR) (600 MHz, CD₃COCD₃), ¹³C-NMR (150MHz, CD₃COCD₃), Heteronuclear Multiple Bond Correlation (HMBC) and ¹H-¹H COSY spectral data are listed in Table 1. ESIMS m/z 335[M+H]⁺, 356 [M+Na]⁺. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) m/z 335.0082 ([M+H]⁺, calcd. 335.0084), 356.9902 ([M+Na]⁺, calcd. 356.9903).

DISCUSSION

Dichlorodiaportinol A (1) m.p. 137–139°C, has a molecular formula of C₁₃H₁₂Cl₂O₆ as determined by HR-ESI-MS m/z 335.0082 ([M+H]⁺, calcd. 335.0084) and m/z 356.9902 ([M+Na]⁺, calcd. 356.9903). Its UV spectrum with absorption maxima at 243, 280 and 326 nm was indicative of a conjugated chromophore. Its IR spectrum with absorption bands at 3,421, 1,685, 1,637, 1,570, and 1,508 cm⁻¹ suggested the presence of hydroxyl, α,β -unsaturated δ -lactone carbonyl, and olefinic functionalities, respectively. These spectra revealed the presence of typical isocoumarin moiety in comparison with known isocoumarins. [10,11] In the ¹H-NMR spectrum, a lowfield, one-proton singlet signal at δ 10.47 (s, 1H, exchangeable) indicated the presence

of a hydrogen-bonded phenolic proton at C-8. [12] Therefore, a pair of meta-coupled doublets ($J = 2.4$ Hz) at δ 6.53 and 6.64, were assigned to H-5 and H-7, respectively. Another aromatic singlet at δ 6.76 (s, 1H), connected to the carbon at δ 108.6 (C-4) in the HSQC spectrum, was ascribed to H-4 of the isocoumarin moiety. A sharp three-proton singlet at δ 3.92 belonging to a methoxy group was connected to C-6 that was determined by the HMBC correlations from 6-OCH₃, H-5, and H-7 to the carbon at δ 169.0 (C-6). The remaining substituent of the isocoumarin was revealed to contain a -CH-CH-CH- fragment by the ¹H-¹H COSY correlations from H-9 to H-10 and H-10 to H-9 and H-11. Two hydroxyls at δ 5.60 (s, 1H, exchangeable) and 5.70 (s, 1H, exchangeable) were, respectively, assigned to be at the positions of C-9 and C-10 by the ¹H-¹H COSY correlations from 9-OH to H-9 and 10-OH to H-10. Therefore, the two remaining chlorine atoms were both connected to C-11. The HMBC correlations from H-9 to C-4 and H-4 to C-9 further demonstrated the connectivity of C-3 and C-9. Hence, the structure of compound 1 was established as 3-(3,3-dichloro-2,3-dihydroxy-propyl)-8-hydroxy-6-methoxy-isochromen-1-one [Figure 1]. We named it Dichlorodiaportinol A, according to its similar structure with the known compound, Dichlorodiaportin. [13]

The relative configuration of compound one was assigned based on the method of J-based configurational analysis and Nuclear Overhauser effect (NOE) data. [14,15] For compound one, H-9 and H-10 were anti due to their large vicinal coupling constant (9.0 Hz). In this case, C-11 and C-3 might be gauche in the threo diastereomer or anti in the erythro diastereomer. Since the NOE interactions between 9-OH and H-11 were also observed, it suggested that 9-OH and H-11 were gauche, and C-11 and C-3 were anti. Accordingly,

Table 1: NMR data for compound 1 (CD₃COCD₃, TMS)

	¹ HNMR	¹³ CNMR (DEPT)	¹ H- ¹ H COSY	HMBC
1		167.6(C)		
3		157.4(C)		
4	6.76(s, 1H)	108.6(CH)		C-3,5,8a, 9
4a		141.0(C)		
5	6.64(d, 2.4Hz, 1H)	103.9(CH)	H-7	C-4,6,7,8a
6		169.0(C)		
6-OCH ₃	3.92(s, 3H)	57.4(CH3)		C-6
7	6.53(d, 2.4Hz, 1H)	102.6(CH)	H-5	C-5,6,8,8a
8		165.2(C)		
8a		102.0(C)		
9	4.40(d, 9.0Hz, 1H)	73.9(CH)	H-10	C-4,10,11
10	4.28(dd, 1.8, 9.0Hz, 1H)	77.7(CH)	H-9,11	C-9,11
11	6.42(d, 1.8Hz, 1H)	77.0(CH)	H-10	C-9,10
9-OH	5.70(s, 1H)		H-9	
10-OH	5.60(s, 1H)		H-10	
8-OH	11.09(s, 1H)			

NMR: Nuclear magnetic resonance, HMBC: Heteronuclear multiple bond correlation, TMS: Tetramethylsilane, HNMR: Proton Nuclear Magnetic Resonance, CNMR: Carbon Nuclear Magnetic Resonance

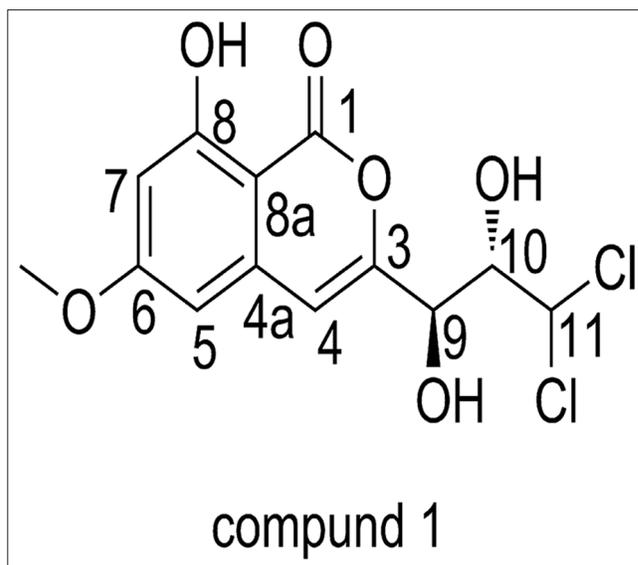


Figure 1: The structure of compound 1

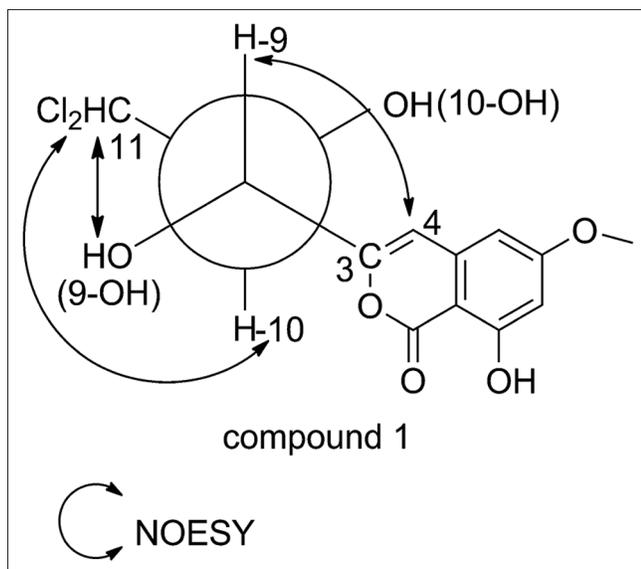


Figure 2: The key NOESY correlations of compound 1

Table 2: Cytotoxicity of compound 1 against MCF-7 and HepG2 cells (IC_{50} , $\mu\text{g/mL}$)

Sample	Cells	
	MCF-7	HepG2
Compound 1	17.8	39.6
Epirubicin ^a	5.3	5.2

a: Used as a positive control

compound one was confirmed as the erythro diastereomer. The key Nuclear Overhauser effect spectroscopy (NOESY) correlations were shown in Figure 2.

Compound one was evaluated for *in vitro* cytotoxic activity against two human cancer cell lines including human breast MCF-7 and human Liver HepG2 by MTT assay^[16,17] using epirubicin (an anticancer drug used widely in the clinic^[18,19]) as positive control. The results are summarized in Table 2. Compound one was moderately active against MCF-7 and HepG2 cells as compared with the standard. Thus, this study suggests that endophytes of *M. bontioides* are worthy of consideration for the development and research of antitumor agents.

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