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Secalonic acid D; A Cytotoxic Constituent from Marine Lichen-derived Fungus *Gliocladium* sp. T31

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Secalonic acid D (SAD) was isolated as the major secondary metabolite of the marine lichen-derived fungus *Gliocladium* sp. T31. Its structure was established on the basic of physico-chemical and spectroscopic data. This is the first report on the isolation of SAD from this fungus, as well as its inhibitory effect on K562 cell cycle and its cytotoxicity against several tumor cell lines *in vitro*.

Key words: Marine lichen-derived, *Gliocladium* sp., Secalonic acid D, Cytotoxicity, Cell cycle inhibition

INTRODUCTION

Marine microorganisms are one of the richest sources of the bioactive secondary metabolites in the marine environment (Tim and Chris, 2004; Bugni and Ireland, 2004), In our screening for cytotoxic agents to human myeloid leukemia K562 cell line, extracts from the metabolite of a marine lichen-derived fungus *Gliocladium* sp. T31 were found to be highly potent. Bioassay-guided fractionation led to the isolation of secalonic acid D (SAD **1**), emodin (**2**), citreorosein (**3**), and isorhodoptilometrin (**4**).

SAD, a mycotoxin, was previously isolated from several food-born fungi (Raymond *et al.*, 1977; Steyn, 1970). Diverse biological activities of SAD have been reported such as a mycotoxin towards chicken and mice embryo (Alex, 1980), an inhibitor of various isozymes of protein kinase C (Ganesh and Chada, 2000; Wang and Poya, 1996) and protein kinase A (Hanumegowda *et al.*, 2002a; Dhulipala *et al.*, 2004) in murine secondary palate development, as well as mouse and human cleft palatal inducing agent (Hanumegowda *et al.*, 2002b; Dhulipala *et al.*, 2004). To the best of our knowledge, there are no studies concerning its cytotoxicity and cell cycle inhibitory activity against human tumor cell lines. In this paper, we report the isolation of SAD from the marine lichen-derived fungus *Gliocladium* sp. and its cytotoxicity and cell cycle inhibition against human tumor cell line *in vitro*.

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MATERIALS AND METHODS

General experimental procedures

Melting points were measured using a Yanaco MP-500D micro-melting point apparatus and were uncorrected. Optical rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on Beckmen DU® 640 spectrophotometer. IR spectra were obtained on a NICOLET NEXUS 470 spectrophotometer in KBr discs. ^{1}H-, ^{13}C-NMR and DEPT spectra and 2D-NMR data were recorded on a JEOL Eclips-600 spectrometer using TMS as internal standard and chemical shifts were recorded as δ values. ESI-MS was measured on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. Semiprepartive HPLC was performed using an ODS column [Shin-pak ODS (H), 20 × 250 mm, 5 μm, 4 mL/min]. Flow cytometry (EPICS XL, Coulter Co., Hialeah, FL, U.S.A.) was used to analyze inhibitory effects on cell cycle.

Marine fungus material

The fungal strain was separated from a marine lichen-derived fungus collected from South Pole, in December 1999, and was frozen and kept at -20°C until fermentation. A voucher of this species was lodged at the Natural Products Chemistry Laboratory, Institute of Marine Drugs and Food, Ocean University of China, China.

Extraction and isolation

The fungal strain was incubated on a rotatory shaker at 120 rpm and 28°C for 9 days in a hundred of 500 mL Erlenmeyer flasks each containing 150 mL of liquid medium composed of manitol 2%, glucose 1%, maltose 2%, monosodium glutamate 1%, corn syrup 0.1%, KH_2PO_4 0.05%, $MgSO_4 \cdot q7H_2O$ 0.03%, yeast extract 0.3% and sea-water then adjusting its pH to 6.5. The culture broth and the mycelium were exhaustively extracted with EtOAc to afford a bioactive crude extract (32.9 g), which was subjected by vacuum liquid chromatography over silica gel H using stepwise gradient elution with the mixtures of light petroleum ether-$CHCl_3$-MeOH to give three bioactive fractions, A, B, C, subsequently. Fraction A was separated by vacuum liquid chromatography over silica gel H using stepwise gradient elution with light petroleum ether-acetone as eluents repeatedly to give emodin (**2**) (11 mg) and isorhodoptilometrin (**4**) (9 mg). Silica gel column chromatography of fraction B eluting with petroleum ether-EtOAc (5:1 to 3:1) gave citreorosein (**3**) (7 mg). Fraction C was recrystalized repeatedly in $CHCl_3$-MeOH to obtain SAD (**1**) (500 mg).

Secalonic acid D (1)

Pale yellow crystal, m.p. 281-283°C, $[\alpha]_D^{25}$ = + 61 (*c* 0.11, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}: 3505 (chelated OH), 1735 (aliphatic ester C=O), 1610 (chelated O-hydroxyl C=O), 1585 (aromatic ring, C=C), 1432, 1232, 1061; ^{1}H-NMR (600 MHz, DMSO-d_6) δ: 13.60 (2H, brs, OH-8, OH-8'), 11.62 (2H, s, OH-1, OH-1'), 7.45 (2H, d, *J* = 8.42 Hz, H-3, H-3'), 6.63 (2H, d, *J* = 8.42 Hz, H-4, H-4'), 3.81 (2H, d, *J* = 9.51 Hz, H-5, H-5'), 3.61 (6H, s, H-13, H-13'), 2.65 (2H, dd, *J* = 8.42 Hz, *J* = 19.80 Hz, Ha-7, Ha-7'), 2.49 (2H, dd, *J* = 6.22 Hz, *J* = 19.80 Hz, Hb-7, Hb-7'), 2.31 (2H, m, H-6, H-6'), 1.03 (6H, d, *J* = 6.22 Hz, H-11, H-11'); ^{13}C-NMR (150 MHz, DMSO-d_6) δ: 186.6 (C-9, C-9'), 178.2 (C-8, C-8'), 170.0 (C-12, C-12'), 158.9 (C-1, C-1'), 158.5 (C-4a, C-4a'), 140.2 (C-3, C-3'), 117.3 (C-2, C-2'), 107.5 (C-4, C-4'), 106.3 (C-9a, C-9a'), 101.7 (C-8a, C-8a'), 85.2 (C-10a, C-10a'), 75.2 (C-5, C-5'), 52.6 (C-13, C-13'), 35.8 (C-7, C-7'), 29.9 (C-6, C-6'), 17.8 (C-11, C-11').

Emodin(2)

Yellow crystal, m.p. 282-284°C; ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 12.08 (1H, s, OH-1), 12.01 (1H, s, OH-8), 11.43 (1H, s, OH-3), 7.48 (1H, brs, H-5), 7.16 (1H, brs, H-7), 7.11 (1H, d, *J* = 2.2 Hz, H-4), 6.58 (1H, d, *J* = 2.2 Hz, H-2), 2.40 (3H, s, CH_3-6); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 189.8 (C-9), 181.4 (C-10), 165.6 (C-1), 164.5 (C-8), 161.4 (C-3), 148.3 (C-6), 135.1 (C-10a), 132.8 (C-4a), 124.2 (C-5), 120.5 (C-7), 113.4 (C-9a), 109.0 (C-8a), 108.8 (C-4), 107.9 (C-2), 21.6 (CH_3-6).

Citreorosein (3)

Yellow crystal, m.p. 282-288°C; ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 12.10 (1H, s, OH-1), 12.07 (1H, s, OH-1), 11.42 (1H, s, OH-1), 7.63 (1H, d, *J* = 1.50 Hz, H-5), 7.25 (1H, d, *J* = 1.50 Hz, H-7), 7.12 (1H, d, *J* = 2.58 Hz, H-4), 6.60 (1H, d, *J* = 2.58 Hz, H-2), 4.61 (2H, d, *J* = 5.46 Hz, CH_2-6); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 189.7 (C-9), 181.4 (C-10), 165.6 (C-1), 164.5 (C-8), 161.5 (C-3), 152.9 (C-6), 135.2 (C-10a), 132.9 (C-4a), 120.8 (C-5), 117.1 (C-7), 114.1 (C-9a), 109.0 (C-8a), 108.8 (C-4), 107.0 (C-2), 62.0 (CH_2-6).

Isorhodoptilometrin (4)

Yellow crystal, m.p. 282-286°C; ^{1}H-NMR (600 MHz, Acetone-d_6) δ: 7.63 (1H, d, *J* = 1.44 Hz, H-5), 7.23 (1H, d, *J* = 2.58 Hz, H-4), 7.18 (1H, d, *J* = 1.44 Hz, H-7), 6.57 (1H, d, *J* = 2.58 Hz, H-2), 4.06 (1H, m, H-2'), 2.82 (2H, d, *J* = 5.82 Hz, H-1'), 1.19 (3H, d, *J* = 6.24 Hz, H-3'); ^{13}C-NMR (150 MHz, acetone-d_6) δ: 190.8 (C-9), 182.6 (C-10), 168.9 (C-1), 166.3 (C-8), 162.7 (C-3), 150.8 (C-6), 136.5 (C-10a), 134.1 (C-4a), 125.4 (C-5), 121.8 (C-7), 114.9 (C-9a), 111.0 (C-8a), 109.3 (C-4), 108.7 (C-2), 68.1 (C-CHOH), 46.3 (C-CH_2), 29.5 (C-CH_3).

Cell cultures

The human lung adenocarinoma (A549), liver adenocarinoma (BEL7402), myeloid leukemia (K562), promyelocytic leukemia (HL-60), and the mouse lymphocytic leukemia (P388) cells were routinely maintained in RPMI-1640 medium supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Cell cycle progression analysis

Flow cytometry assay was performed according to the previously reported method (Dhulipala *et al.*, 2004). Cells were plated at a density of 20×10^5 cells/mL in the absence or presence of the indicated concentrations of SAD, and cultured at 37°C for 24 h. The treated cells were washed twice in PBS and stained with 150 μL propidium iodide (PI) in water solution at 4°C for 30 min under lightproof condition, and the cell cycle distribution was determined by flow cytometry analysis using the computer software WinCycle (Coulter).

In vitro cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) assay was performed according to the previously reported method (Mosmann, 1983). The inhibition reates (IR %) were calculated using OD mean values from IR % = $(OD_{control} - OD_{sample})/OD_{control}$. The IC_{50} value, which was defined as the concentration of sample needed to reduce a 50% of absorbance relative to the vehicle-treated control, was determined using the Bliss method. The same experiment was repeated independently three times to obtain a mean IC_{50} and its standard deviation.

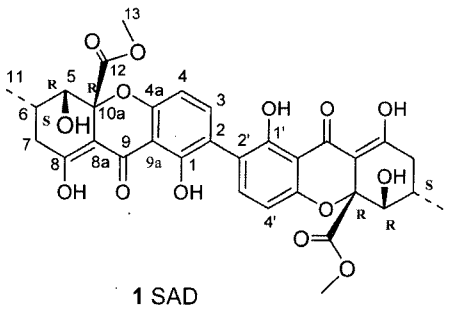
Analysis of DNA fragmentation

The method of DNA fragmentation analysis was used as described (Viola *et al.*, 2000). The cells were, briefly, incubated at 50°C with lysis buffer containing 10 mM EDTA, 0.5% *N*-laurylsarcosine and 0.5 mg/mL proteinase K in 50 mM Tris-HCl (pH 8.0), then added RNase to a concentration of 0.25 mg/mL. DNA was extracted with phenol/chloroform and phenol/chloroform/isopentanol twice. The extract was diluted with Tris EDTA (TE) buffer and centrifuged at 13000 g for 10 min. The precipitated DNA was dissolved in TE buffer. The sample buffer and 0.1% ethidium bromide (EB) were electrophoresed on 1.5% agarose gel with Tris-borate EDTA buffer. DNA was visualized under UV light.

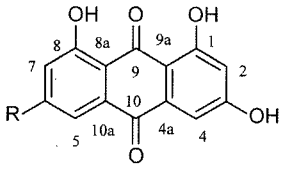
RESULTS AND DISCUSSION

Bioassay-guided fractionation of a cude EtOAc extract from the fungus *Gliocladium* sp. T31 through repeated silica gel column chromatography and recrystalization led to the isolation of compounds **1**-**4**. By comparison of the spectral and physicochemical data with those reported previously, compounds **2**, **3**, and **4** were identified as emodin (**2**) (Lu *et al.*, 1983), citreorosein (**3**) (Bachanan *et al.*, 1998), and isorhodoptilometrin (**4**) (Jacqueb, 1976), respectively.

Compound **1** was obtained as a pale yellow crystal in $CHCl_3$. Its molecular formula, $C_{32}H_{30}O_{14}$, was deduced by ESI-MS, ^1H- and ^{13}C-NMR spectra. Its IR spectrum showed strong absorption at 3505, 1735, 1610, and 1595 cm^{-1}. The NMR spectrum only showed the presence of fifteen protons and sixteen carbons, implying that **1** was a symmetrical dimer. Analysis of ^1H, ^{13}C, DEPT, and HMQC spectra revealed that half of the molecule, $C_{16}H_{15}O_7$, possessed one conjugated ketone carbonyl δc 186.6 (C-9), one ester carbonyl 170.0 (C-12), seven quaternary carbons δc 178.2 (C-8), 158.9 (C-1), 158.5 (C-4a), 106.3 (C-9a), 101.7 (C-8a), 85.2 (C-10a), and 117.3 (C-2), two sp^2 methines (δc 140.2, δ_H 7.45; δc 107.5, δ_H 6.63), one sp^3 methines (δc 29.9, δ_H 2.31), one oxymethine (δc 75.2, δ_H 3.81), one methylene (δc 35.8, δ_H 2.65 and 2.49), one methoxyl (δc 52.6, δ_H 3.61), one methyl group (δc 17.8, δ_H 1.03), and two proton signals of chelated phenolic hydroxyl



1 SAD



2 R = CH_3
3 R = CH_2OH
4 R = CH_2CH (OH) CH_3

Fig. 1 Structures of secalonic acid D (**1**), emodin (**2**), citreorosein (**3**), and isorhodoptilometrin (**4**)

groups (δ_H 13.60 and 11.62) were also observed. COSY correlations revealed the connectivity of C-5 to C-6 to C-7, and the hexahydroxanthone structure was established on the basis of the HMBC correlations. An intense correlation between the chelated phenolic hydroxyl proton (OH-1, δ_H 11.62) and the quaternary carbon at δc 117.3 placed this carbon at C-2, hence compound **1** was a C-2-C-2' dimer. Compared with the previously reported data (Raymond *et al.*, 1977), the structure was assigned to be secalonic acid D. Furthermore, **1** is an optically active compound and has specific rotation of + 61 (*c* 0.11, $CHCl_3$), similar to the reported value (+64, *c* 0.14, $CHCl_3$) for SAD (Steyn, 1970), which confirmed its structure.

The cytotoxicity assay of SAD was carried out against four tumor cell lines P388, A549, K562, and BEL7402 according to the MTT assay (Mosmann, 1983) and the IC_{50} values were 0.03, 0.26, 5.76, and 15.50 μM, respectively. SAD exhibited strong cytotoxicity to K562, A549, and P388 cells and very weak on BEL7402 cell, which suggested that SAD had some selectivity to different tumor cells. Up to date there are no detailed reports on cytotoxicity of SAD against these tumor cell lines. Although its teratogenic effects on humans have not been studied, the only malformation in fetal mice producing cleft palatae (CP) suggests that as little as 30 mg SAD/kg body weight, intraperitoneally, can induce approximately 50% incidence of CP in the offspring (Ganesh and Chada, 2000). Compared with the teratogenic dose in mice, the findings we obtained towards P388, A549, and K562 cells were at least 50-fold lower than that of ip in mice by dose conversion.

In addition, we studied the effect of SAD on the cell

cycle distribution of K562 cells by flow cytometry assay. K562 cells were treated with SAD at the concentrations of 1-10000 nM for 24 h. The cell cycle analysis revealed that low concentrations of SAD (1-10 nM) did not affect the distribution of K562 cells in the various phases of the cell cycle. Moderate concentrations of SAD (0.1-1.0 μM) did, however, alter the distribution of these cells in the cell cycle. The findings was summarized in Fig. 2 and Table I. With the increment of SAD concentration, the cells in G_0/G_1 phase gradually increased and the ones in S phase decreased correspondingly. Up to 0.5 μM SAD, the cells in S phase decreased almost a half, while the cells in the G_0/G_1 phase increased twice, which indicated that SAD induced a G_0/G_1 block in a concentration-dependent fashion. Work by Dhulipala and colleagues (Dhulipala *et al.*, 2004) had reported that SAD inhibits the progression of human embryonic patatal mesenchymal cells *in vitro* from G_1 to S phase of the cell cycle only treated with IC_{50}

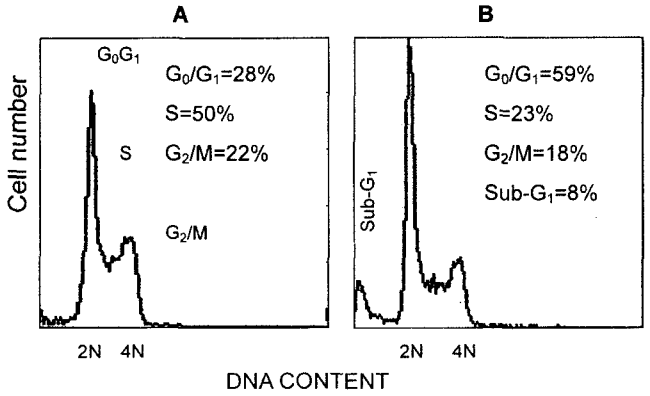


Fig. 2. DNA histogram of K562 cells with SAD. K562 cells were treated with SAD 1.0 μM for 24 h and the DNA content was analyzed by flow cytometry. (A) Untreated control cells. (B) SAD 1.0 μM treated cells, showing a G_0/G_1 block and a subdiploid peak.

Table I. The effect of SAD at 24 h on the cell cycle distribution of the K562 cells

Concentration (μM)	Distribution (%)			
	Sub-G_1	G_0/G_1	S	G_2/M
0.000	0.1	28.3	50.1	21.6
0.001	0.1	26.8	51.2	23.4
0.010	0.1	25.6	54.8	19.6
0.100	2.1	41.3	43.0	18.2
0.500	2.8	53.8	30.1	17.2
1.000	7.7	59.4	23.3	18.5
5.000	10.4	56.4	26.9	20.2
10.00	25.2	52.2	27.2	19.9

The K562 cells were treated for 24 h at the indicated concentrations of SAD. The percentage of cells in each phase of the cell cycle was analyzed with the computer software WinCycle (Coulter). This protocol was followed three times. Each time findings were within 5%.

of SAD for 48 h. The present study showed SAD induced a G_0/G_1 block in a concentration-dependent manner towards K562 cells *in vitro*.

Exposure to higher level of SAD (5.0-10.0 μM) for 24 h, except the G_0/G_1 arrest, a large increment of sub-G_1 population or a subdiploid peak was also observed. To assess whether the cells underwent active apoptosis, we diagnosed the "DNA ladders" by agarose gel electrophoresis. Negative results in the DNA fragmentation analysis suggested that the cell death caused by higher levels of SAD is unrelated to apoptosis.

In our investigation, a potent antitumor component, SAD, was found from marine lichen-derived fungus *Gliocladium* sp. T31 by a bioassay-guided separation procedure for the first time. The cytotoxic effects of SAD were preliminarily evaluated, as shown above. SAD exhibited potent cytotoxicity against K562, A549, and P388 cells and weak effect on BEL7402 cell. Notably, SAD displayed significant selectivity to different tumor cells and its effective dose IC_{50} is at least 50-fold lower than that of teratogenic effect in mice. Furthermore, SAD obviously inhibited the cell cycle of K562 cells at the G_0/G_1 phase in a concentration-dependent manner. In conclusion, the inhibiting ability of SAD to the proliferation of several tumor cells as well as blocking the progression of K562 cell cycle suggested that its potential of inhibiting human tumor cells needs to be elucidated in detail.

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REFERENCES

Alex, C., Wallace, A. H., and Ronald, F. V., Production and biological activity of Secalonic D. *Applied and Environmental Microbiology,* 39, 285-287 (1980).

Bachanan, M. S., Melvyn, G., and Alberto, G., Pigments of fungi. XLVI. new dimeric dihydroanthracenones of the flavomannin type from an australian toadstool of the genus *Dermocybe. J. Chem.*, 51, 103-110 (1998).

Bugni, T. S. and Ireland, C. M., Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Nat. Prod. Rep.,* 21, 143-163 (2004).

Dhulipala, V. C., Welshons, W. V., and Reddy, C. S., Inhibition of human embryonic patatal mesenchymal cell cycle by secalonic acid D: a probable mechanism of its cleft palate induction. *Orthod Craniofacial Res.,* 7, 227-236 (2004).

Ganesh, B. and Chada, S. R., Novel mechanism of protein kinase C inhibition involving the pseudosubstrate region by secalonic acid D *in vitro. Toxicology and Applied Pharmacology,* 163, 86-93 (2000).

Hanumegowda, U. M., Barbara, M. J., Wade, V. W., and Chada, S. R., Selective inhibition of murine palatal mesenchymal cell proliferation *in vitro* by secalonic acid D. *Toxicol. Sci.,* 66, 159-165 (2002b).

Hanumegowda, U. M., Dhulipala, V. C., and Reddy, C. S., Mechanism of secalonic acid D-induced inhibition of transcription factor binding to cyclic AMP response element in the developing murine palate. *Toxicol. Sci.,* 70, 55-62 (2002a).

Jacqueb, P. B., Reaction of keten acetals. Part IV: Total syntheses of anthraquinones (±)-nalyiovensin, (±)-isorhodoptilometrin, and (±)-rhodopilometrin [J]. *JCS Perkin I*, 613-619 (1976).

Lu, Y. H., Wang, Z. T., Xu, L. S., and Wu, Z. B., Three Anthraquinones Isolated from *Astertataricus. Journal of Chinese Pharmaceutical Science,* 12, 112-113 (2003).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods,* 65, 55-63 (1983).

Raymond, A., George, B., Brunhilde, K., and Arnold, L. D., *Secalonic acid D and F are toxic metabolites of* Aspergillus aculeatus. *J. Org. Chem.*, 42, 352-353 (1977).

Steyn, P. S., The isolation, structure and absolute configuration of secalonic acid D, the toxic metabolite of *Penicillium Oxalicum. Tetrahedron,* 26, 51-57 (1970).

Tim, S. B. and Chris, M. I., Marine-derived Fungi: A Chemically and Biologically Diverse Group of Microorganisms. *Nat. Prod. Rep.,* 21, 143-163 (2004).

Viola, A., Lutz, N. W., Maroc, C., Julliard, M., and Cozzone, P. J., Metabolic effects of photodynamically induced apoptosis in an erythrolukemic cell line. A (31)P NMR spectroscopic study of Victoria-Blue-BO-sensitized TF-1 cells. *Int. J. Cancer*, 85, 733-739 (2000).

Wang, B. H. and Poya, G. M., The fungal teratogen secalonic acid D is an inhibitor of protein kinase C and of cyclic AMP-dependent protein kinase. *Planta Med.*, 62, 111-114 (1996).