School of Basic Medical Science[1], Ningxia Medical University, Yinchuan; School of Traditional Chinese Materia Medica[2], Shenyang Pharmaceutical University, Shenyang; College of Chemical Engineering[3], Qingdao University of Science & Technology, Qingdao; The First Institute of Oceanography[4], State Oceanic Administration of China, Qingdao; Research Center of Medical Science and Technology[5], Ningxia Medical University, Yinchman, P.R. China

Cytotoxic piperazine-2,5-dione derivatives from marine fungus *Gliocladium* sp.

Yao Yao[1,5], Li Tian[3,4], Juan Li[1], Jiaqing Cao[2], Yuehu Pei[2]

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Yuehu Pei, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, 110016, P.R. China
peiyueh@vip.163.com

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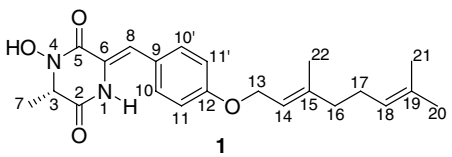
Two new piperazine-2,5-dione derivatives, gliocladride A (**1**) and B (**2**), along with deoxymycelianamide (**3**) were isolated from the mycelial mass of marine fungus *Gliocladium* sp. Their structures were established on the basis of spectral data, and the stereochemical assignments were made by chiral HPLC analysis of the hydrolyzed compounds and optical rotation comparison with known compound. Their cytotoxic activity was tested on three cancer cell lines, HL-60, U937 and T47D by MTT assay. As a result, gliocladride A (**1**) and B (**2**) showed moderate cytotoxic activity against the three cell lines with IC_{50} values from 11.60 μg/ml to 52.83 μg/ml, while deoxymycelianamide (**3**) showed strong cytotoxic activity against U937 cell line with IC_{50} values of 0.785 μg/ml.

1. Introduction

Marine microorganisms are a promising source for novel antitumor agents (Fenical 1993). Marine derived fungi also represent potential for the discovery of new cytotoxic metabolites (Bugni et al. 2004). To discover new cytotoxic compounds, we have investigated several marine microorganisms (Zhang et al. 2004; Huang et al. 2006a, 2006b; Sun et al. 2006a, 2006b). In our continuous study, the fungus *Gliocladium* sp. was isolated from sea mud collected in Rushan, Shandong province, China, and then be cultivated and extracted. The primary bioassay of the acetone extract from the mycelial mass showed strong inhibition against *Alternaria solani*. Repeated column chromatography on the acetone extract afforded three piperazine-2,5-dione derivatives (**1**–**3**). We report here the structure elucidation of the two new compounds (**1**–**2**) and the cytotoxic activities of all compounds on three cancer cell lines, HL-60, U937 and T47D.



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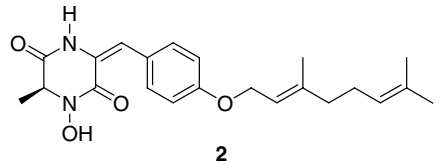

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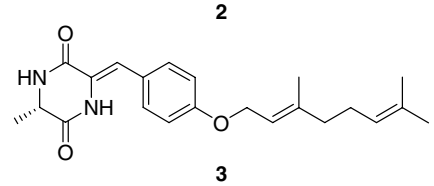

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2. Investigations, results and discussion

Gliocladride A (**1**) was isolated as a white solid, and it's molecular formula was determined to be $C_{22}H_{28}N_2O_4$ from its HR-FAB MS at m/z 385.2125 ($[M + H]^+$, calcd. for 385.2127), indicating 10 degrees of unsaturation. The UV bands at 225, 315 nm and IR absorptions at 3310, 2921, 1688, 1606, 1521, 1442 and 1251 cm^{-1} suggested the presence of hydroxyl, amide and aromatic groups. General analyses of ^1H NMR (600 MHz, DMSO-d_6), ^{13}C NMR (150 MHz, DMSO-d_6) and HMQC signals gave some important information about the structure. Four aromatic proton signals δ7.45 (2 H, d, J = 8.7 Hz, H-10,10′), 6.96 (2 H, d, J = 8.7Hz, H-11,11′) were typical for a 1,4-disubstituted aromatic ring; a vinyl singlet at δ6.74 (1 H, s, H-8) and it's HMBC correlation with δ131.0 (C-10) indicated the presence of a benzylidene unit. Three CH_3 groups at δ1.71 (3 H, s, H-22), 1.64 (3 H, s, H-20), 1.57 (3 H, s, H-21), three CH_2 groups at δ4.57 (2 H, d, J = 6.6 Hz, H-13), 2.09 (2 H, m, H-17), 2.04 (2 H, m, H-16) and two vinyl groups at δ5.43 (1 H, t, J = 6.6 Hz, H-14), 5.08 (1 H, t, J = 6.6 Hz, H-18) were attributable to a geranyloxy side chain, of which the three methyl resonance assignments were made by HMBC and NOESY correlations. The methyl signal at δ1.71 (3 H, s, H-22) was positioned at C-15 on the basis of HMBC correlation between H-22 and vinyl carbons at δ140.5 (C-15) and

δ119.6 (C-14). The signal at δ1.64 (3 H, s) was assignment to be H-20 as the NOE correlation between H-20 and vinyl proton signal δ5.08 (1 H, t, J = 6.6 Hz, H-18), while NOE correlation between δ1.57 (3 H, s, H-21) and δ5.08 (1 H, t, J = 6.6 Hz, H-18) was not observed. The remaining ^{1}H NMR (600 MHz, DMSO-d_6) resonances at δ9.96 (1 H, s, NH-1), δ4.31 (1 H, q, J = 6.8 Hz, H-3), δ10.22 (1 H, s, NH−OH), δ1.45 (1 H, d, J = 6.8 Hz, H-7) and ^{13}C NMR (150 MHz, DMSO-d_6) resonances at δ166.3 (C-2), δ60.0 (C-3), δ157.3 (C-5), δ123.4 (C-6), δ17.1 (C-7) were attributable to a 3-methyl-2,5-diketonpiperazine unit, which was supported by the HMBC correlations between D_2O exchangeable proton at δ9.96 (1 H, s, NH-1) and δ166.3 (C-2), δ123.4 (C-6), and between δ4.31 (1 H, q, J = 6.8 Hz, H-3) and δ166.3 (C-2), δ157.3 (C-5), δ17.1 (C-7). The other D_2O exchangeable proton at δ10.22 (1 H, s, N−OH) had weak correlations with δ60.0 (C-3) and δ157.3 (C-5). Thus, the remaining one hydroxyl group accounted from the molecular composition was positioned on N-4. Then we got three units: a benzylidene unit, a geranyloxy side chain and a 3-methyl-4-*N*-hydroxyl-2,5-diketonpiperazine unit. The connection of these three units was based on HMBC correlation. The HMBC correlation between δ6.74 (1 H, s, H-8) and δ123.4 (C-6), 125.7 (C-9) indicated that the benzylidene unit was connected with the piperazine-2,5-dione ring by C-6. The correlation from δ4.57 (2 H, d, J = 6.6 Hz, H-13) to δ158.4 (C-12) showed that the geranyloxy side chain was connected with the benzylidene unit by C-12.

The configuration of double bonds was determined by NOESY experiment. NOE between aromatic proton at (7.45 (2 H, d, J = 8.7 Hz, H-10, 10′) and proton at (9.96 (1 H, s, NH-1) confirmed the Z-configuration of $\Delta^{6,8}$ double bond in the benzylidene unit; NOE between methyl protons at (1.71 (3 H, s, H-22) and methene protons at (4.57 (2 H, d, J = 6.6 Hz, H-13) confirmed the E-configuration of $\Delta^{14,15}$ double bond in the geranyl group. To determine the absolute configuration of C-3, acid hydrolysis and chiral HPLC analysis of **1** were done. The Ala in **1** was defined as L-Ala, thus C-3 was S-configuration as shown in Fig. 1, which was consistent with the optical rotation comparison. It's optical rotation ($[\alpha]_D$-150) showed the same sign as that of 3S-mycelianamide ($[\alpha]_D$-217) (Brich et al. 1956).

Gliocladride B (**2**) was also a white solid, which had the same molecular formula as gliocladride A (**1**) from its HR-FAB MS at m/z 385.2122 ($[M + H]^+$, calcd. for 385.2127). The UV bands at 224, 315 nm, IR absorptions at 3310, 2920, 1687, 1605, 1521, 1442 and 1250 cm^{-1} and its NMR spectrums were quite similar with those of **1** (Table 1). Main difference was in the NOESY spectrum: NOE correlation between (δ6.56 (1 H, s, H-8) and (δ9.62 (1 H, s, NH-1) was observed instead of the correlations between (δ7.36 (2 H, d, J = 8.7 Hz, H-10, 10′) and (9.62 (1 H, s, NH-1). Thus the $\Delta^{6,8}$ double bond in **2** was determined to be E-configuration. The configuration of C-3 was assumed to be S due to its optical rotation ($[\alpha]_D$-126) which had the same direction as **1**, thus gliocladride B was decided to be a cis-trans-isomer of gliocladride A as shown in Fig. 2.

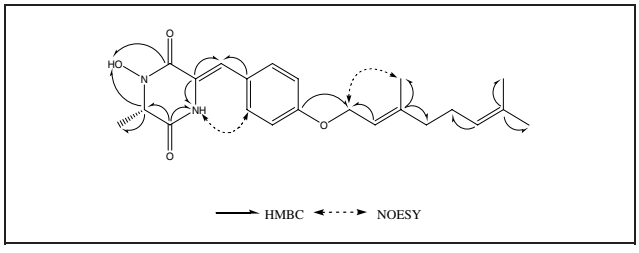

Fig. 1: Selective HMBC and NOESY correlations of gliocladride A (1)

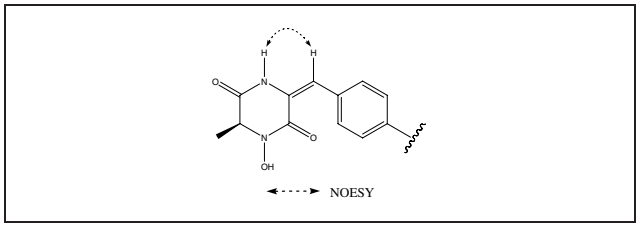

Fig. 2: Selective NOESY correlation of gliocladride B (2)

Table 1: ^{1}H and ^{13}C NMR data of compounds 1 and 2 in DMSO-d_6[a]

Position	1		2	
	δ_C	δ_H (J_{Hz})	δ_C	δ_H (J_{Hz})
1		9.96 (s)		9.62 (s)
2	166.3		162.0	
3	60.0	4.31(q, 6.8)	59.3	4.40 (q, 6.8)
4		10.22 (s)		10.49 (s)
5	157.3		157.8	
6	123.4		126.0	
7	17.1	1.45 (d, 6.8)	16.8	1.45 (d, 6.8)
8	115.2	6.74 (s)	117.9	6.56 (s)
9	125.7		125.1	
10, 10′	131.0	7.45 (d, 8.7)	132.5	7.36 (d, 8.7)
11, 11′	114.9	6.96 (d, 8.7)	113.7	6.84 (d, 8.7)
12	158.4		158.7	
13	64.5	4.57 (d, 6.6)	64.5	4.56 (d, 6.6)
14	119.6	5.43 (t, 6.6)	119.7	5.42 (t, 6.6)
15	140.5		140.5	
16	38.9	2.04 (m)	38.9	2.04 (m)
17	25.9	2.09 (m)	25.9	2.08 (m)
18	123.9	5.08 (t)	123.9	5.08 (t)
19	131.2		131.1	
20	25.6	1.64 (s)	25.6	1.63 (s)
21	17.7	1.57 (s)	17.7	1.57 (s)
22	16.5	1.71 (s)	16.5	1.71 (s)

[a] All assignments are based on HSQC and HMBC experiments

Compound **3** was found to be identical to deoxymycelianamide by spectroscopic data analysis and comparison of its chemical and physical properties with those reported in the literature (Gallina et al. 1966, 1968). This is the first time to report its cytotoxic activity.

The cytotoxicity of compounds **1–3** was evaluated against three cancer cell lines, HL-60, U937 and T47D, in an MTT assay (Mosmann 1983). Vincristin was tested as a reference. Structure-function relationships of these compounds from the activity test results (Table 2) need to be discussed. The exist of *N*−OH may decrease the cytotoxic activity, while the configuration of $\Delta^{6,8}$ double bond seems to have no significient effect.

Table 2: The results of cytotoxic activity test[a]

	HL-60	U937	T-47D
1	17.87	12.80	42.80
2	19.86	11.60	52.83
3	2.02	0.79	30.51
Vincristin	2.46	1.67	12.57

[a] IC_{50} values, μM, cells were treated for 72 h

3. Experimental

3.1. General Procedures

Optical rotations were measured on a Perkin-Elmer 241MC polarimeter. UV spectra were performed with a Shimadzu UV260 spectrometer. IR spectra were recorded on a Thermo Nicolet Nexus 470 FT-IR spectrometer. NMR spectra were recorded on a Bruker-ARX-600 spectrometer (^{1}H at 600 MHz and ^{13}C at 150 MHz). HR-FAB MS spectra were taken with a Q-trap LC-MS-MS system using turbo ionspray source. Colume chromatography was carried with silica gel (200–300 mesh) obtained from Qingdao Marine Chemistry Co. Ltd., Qingdao, P.R. China.

3.2. Fungus material

The fungus strain was isolated from sea mud collected in Rushan, Shandong province, China, in May of 2004, and identified as *Gliocladium* sp. by Prof. Li Tian. A voucher specimen (No.CAAN045011) is deposited in the key laboratory of Marine Biology of State Oceanography Administration, China.

3.3. Cultivation and extraction

The strain was cultured on seed medium at 24° on a rotary shaker for 9 days. The culture medium contained potato decoction 200 ml, sea mud extract 20 ml, peptone 2 g, dextrose 15 g, NaCl 12 g, $MgCl_2 \cdot 6\,H_2O$ 1.1 g, KCl 0.1 g, and distilled water 1000 ml. On the tenth day, the fermentation broth, including cells, was harvested and then centrifugated to separate mycelial mass from aqueous layer. The mycelial mass was exhaustively extracted with acetone (six times) to get a crude extract (26 g).

3.4. Isolation and characterization

The extract was subjected to gradient elution in petroleum ether/acetone (100 : 1 to 1 : 1) on a silica gel column to give a series of fractions. The seventh fraction (5 : 1) was chromatographed over Sephadex LH-20 column (Pharmadex, $CHCl_3$/MeOH 1 : 1) and further purified on reversed-phase silica gel (Chromatorex C_{18}, MeOH/H_2O 7 : 3), to give compound **3** (42.0 mg). The eighth fraction (10 : 3) was chromatographed over Sephadex LH-20 column (Pharmadex, $CHCl_3$/MeOH 1 : 1) and further purified on reversed-phase prep-HPLC (Shimazu, ODS-C_{18}, MeOH/H_2O 7 : 3), to afford compounds **1** (30.2 mg) and **2** (12.6 mg).

3.5. Gliocladride A (1)

White solid; $[\alpha]_D^{20}$-150°(c 0.05, CH_3OH); UV λ_{max} (CH_3OH, log ε) nm 225 (3.94), 315 (4.02); IR (KBr) ν_{max} cm^{-1} 3310, 2921, 1688, 1606, 1521, 1442, 1251 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; EI-MS m/z: 384 $[M]^+$, 368, 248, 232, 217, 133, 81, 69 (base); HR-FAB MS m/z 385.2125 ($[M + H]^+$, calcd. for 385.2127).

3.6. Gliocladride B (2)

White solid; $[\alpha]_D^{20}$-66°(c 0.05, CH_3OH); UV λ_{max} (CH_3OH, log ε) nm 224 (3.92), 315 (4.06); IR (KBr) ν_{max} cm^{-1} 3310, 2920, 1687, 1605, 1521, 1442, 1250 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; EI-MS m/z: 384 $[M]^+$, 368, 248, 217, 133, 81, 69 (base); HR-FAB MS m/z 385.2122 ($[M + H]^+$, calcd. for 385.2127).

3.7. Acid hydrolysis and chiral amino acid analysis

Compound **1** (2.0 mg) was hydrolyzed by heating the sample in a seal vial at 120 °C for 22 h in 6 N HCl, and then dried under vacuum. The hydrolysate was eluted from a C18 column (Dikma) using MeOH/H_2O (10 : 90). The elute was dried under vacuum and reconstituented with 100 μL of H_2O prior to analysis [CHIRAL PAK CR(+), 4.6 × 150 mm; detection: UV 200 nm; injected amount: 5 nmol; mobile phase: pH 1.5 $HClO_4$ in H_2O, flow rate 0.4 ml/min]. The hydrolysate was chromatographed alone and co-injected with standards to confirm assignment. Retention time of the in **1** was 4.66 min, which was identical to the authentic L-Ala. The standard retention time of the D-Ala was 3.54 min.

3.8. Cytotoxic activity test

The three cancer cell lines, HL-60, U937 and T47D, were obtained from the American Type Culture Collection (ATCC), and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), in a humidified atmosphere of 5% CO_2 in air, at 37 °C. Cell inhibition were measured by MTT assay (Mosmann 1983), and all experiments were performed five times.

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