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A new piperazine-2,5-dione from the marine fungus *Gliocladium* sp.

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A new piperazine-2,5-dione, named gliocladride, was isolated from marine fungus *Gliocladium* sp. The structure was established on the basis of spectral data, and the stereochemical assignments were made by ^{1}H NMR spectrum and chiral HPLC of the hydrolyzed compound. Gliocladride showed a cytotoxic effect with an IC_{50} value of 3.86 μg/ml against human A375-S2 melanoma cell line.

Marine microorganisms have proven to be a promising source for the production of novel antitumor agents (Fenical 1993). Marine derived fungi also represent potential for the discovery of new cytotoxic metabolites (Bugni and Ireland 2004). To discover new cytotoxic compounds, we have investigated the chemical constituents of marine fungus *Gliocladium* sp., and report here the isolation, characterization and biological activity of a new piperazine-2,5-dione, gliocladride (**1**).

Compound **1**, a white solid, was formulated as $C_{22}H_{30}N_2O_3$ from its HR-FAB MS at m/z 371.2324 ($[M + H]^+$, calcd. for 371.2335). General analyses of ^{1}H NMR, ^{13}C NMR and HMQC signals showed some important information about the structure. Two carbonyls (δ 167.78, 166.01), two NH groups (δ 8.07, 7.99) and two α-CH groups (δ 55.56, 49.81) indicated the presence of a piperazine-2,5-dione ring. Four aromatic proton signals δ 7.02 (2 H, d, J = 8.5 Hz), 6.82 (2 H, d, J = 8.5 Hz) were typical for a 1,4-disubstituted aromatic ring. Three CH_3 groups at δ 1.66 (3 H, s), 1.62 (3 H, s), 1.55 (3 H, s), three CH_2 groups at δ 4.47 (2 H, d, J = 6.3 Hz), 2.04 (2 H, m), 1.99 (2 H, m) and two CH groups at δ 5.36 (1 H, t, J = 6.3 Hz), 5.05 (1 H, t, J = 6.6 Hz) were attributable to an *O*-geranyl residue. These three residues were confirmed by HMBC experiment. In the HMBC spectrum, the two-bond correlations between δ 0.50 (3 H, d, J = 6.9 Hz, H-7) and δ 49.81 (C-3) indicated that one of the amino acid units in the structure was Ala. The two-bond correlations between δ 2.75 (1 H, dd, J = 13.5, 4.8 Hz, H-8b) and δ 55.56 (C-6), 127.86 (C-9) indicated that the piperazine-2,5-dione ring was connected with the aromatic ring through C-8. The three-bond correlations from δ 4.47 (2 H, d, J = 6.3 Hz, H-13) to δ 157.40 (C-12) showed that the *O*-geranyl residue was connected with the aromatic ring by C-12. Therefore the other amino acid unit was identified to be Tyr. Then, a NOESY experiment was conducted, NOE between methyl protons at δ 1.66 (3 H, s, H-22) and methene protons at δ 4.47 (2 H, d, J = 6.3 Hz, H-13) confirmed the E-configuration of $\Delta^{14,15}$ double bond in the geranyl group, and the structure of **1** was determined to be cyclo-*O*-geranyl-tyrosyl-alanine as shown in Fig.1.

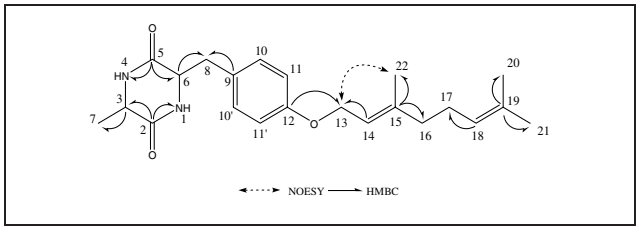

Fig. 1: Selective HMBC and NOESY correlations of gliocladride

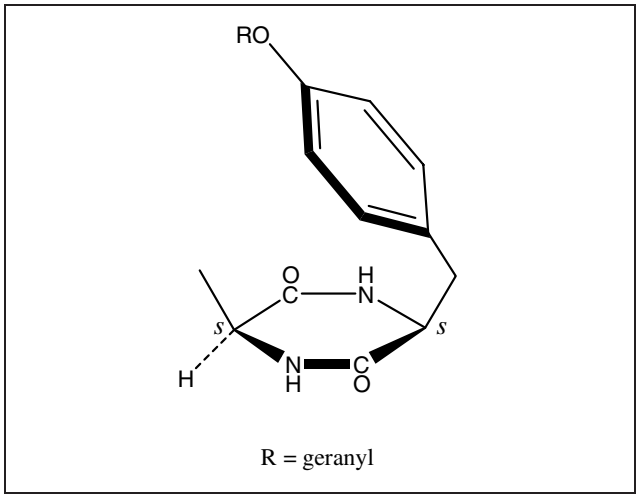

Fig. 2: The stereochemistry of gliocladride

Information on the stereochemistry could be obtained from the ^{1}H NMR spectrum as follows: the Cβ methyl protons of the alanyl residue (H-7) resonated at higher field than that expected from the free amino acid, and this was consistent with previous findings (Kopple and Marr 1967) concerning piperazine-2,5-diones derived from aromatic amino acids. These protons underwent a strong anisotropic shielding effect due to the neighboring aromatic ring, indicating a cis configuration of the two amino acid side chains. To determine the absolute configuration, acid hydrolysis and chiral HPLC analysis of **1** were done. The Ala in **1** was defined as L-Ala, thus C-3 and C-6 were all S-configuration as shown in Fig.2.

The cytotoxicity of gliocladride was evaluated against A375-S2 melanoma cell line using MTT assay (Mosmann 1983), which showed moderate effect with an IC_{50} value of 3.86 μg/ml. Vincristin was tested as a reference, which had an IC_{50} value of 1.22 μg/ml.

Experimental

1. Apparatus

NMR spectra were recorded on Bruker-ARX-600 spectometer (^{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.

2. Fungus material

The fungus strain was isolated from sea mud collected in Rushan, Shandong province, China, in May 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. Cultivation and extraction

The strain was cultured on seed medium at 24 °C 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, $MgCl_2$ 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 centrifuged to separate mycelial mass from aqueous layer. The mycelial mass was exhaustively extracted with acetone to get a crude extract (26 g).

4. Isolation and characterization of 1

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 **1** (12.2 mg). Compound **1**: ^{1}H NMR (600 MHz, DMSO-d_6)/^{13}C NMR (150 MHz, DMSO-d_6): 7.99 (1 H, s, H-1), 167.78 (C-2), 3.61 (1 H, q, J = 6.9 Hz, H-3)/49.81, 8.07 (1 H, s, H-4), 166.01 (C-5), 4.09 (1 H, m, H-6)/55.56, 0.50 (3 H, d, J = 6.9 Hz, H-7)/19.81, 3.04 (1 H, dd, J = 13.5, 6.4 Hz, H-8a)/37.52, 2.75 (1 H, dd, J = 13.5, 4.8 Hz, H-8b), 127.86 (C-9), 7.02 (2 H, d, J = 8.5 Hz, H-10,10′)/131.40, 6.82 (2 H, d, J = 8.5 Hz, H-11,11′)/114.38, 157.40 (C-12), 4.47 (2 H, d, J = 6.3 Hz, H-13)/64.36, 5.36 (1 H, t, J = 6.3 Hz, H-14)/119.90, 139.99 (C-15), 1.99 (2 H, m, H-16)/39.03, 2.04 (2 H, m, H-17)/25.92, 5.05 (1 H, t, J = 6.6 Hz, H-18)/123.89, 131.13 (C-19), 1.62 (3 H, s, H-20)/25.60, 1.55 (3 H, s, H-21)/17.67, 1.66 (3 H, s, H-22)/16.45; for selected HMBC and NOE correlations, see Fig. 1.

5. Acid hydrolysis and chiral amino acid analysis

Compound **1** (2.0 mg) was hydrolyzed by heating the sample in a seales 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 reconstituted 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 Ala in **1** was 4.67 min, which was identical to the authentic L-Ala. The standard retention time of d-Ala was 3.54 min.

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