lism and cessation of division within 7 days. All cultures were seeded at a cell density of $1 \times 10^4/cm^2$ unless otherwise noted, and were allowed to proliferate, undisturbed, for 7 days. Various concentrations of dendroflorin were added 24 h before examination with solvent as control, unless otherwise stated. The concentration of DMSO in cultures was less than 0.3% (v/v).

3. *MTT assay*

The method described by Chiba et al. (1998) was used in this study. In brief, the cells were seeded into 96 well microplates at a density of 2.5×10^3 cells/0.2 ml per well. 20 h later, the cells were in a subconfluent state and were exposed to dendroflorin, followed by 24 h incubation with 5% CO_2 at 37 °C. 25 μl MTT/PBS solution [3-(4,5-dimethythiazolzyl)-2, 5-diphenyl tetrazolium tromide, 10 mg/ml (Sigma)] was then added, and 4 h later, the formazan precipitate was dissolved in 0.2 ml DMSO. The absorbance values of each well were assayed spectrophotometrically at 490 nm on the spectrophotometer using a Microplate Reader (BIO-TEK, Rockville, MA, USA).

4. *Flow cytometry analysis*

Flow cytometry was introduced to measure the DNA contents and intracellular ROS levels using propidium iodide and DCFH-DA respectively. Cells were plated at a density of 1×10^5 cells/10 ml cell solution in 100 mm diameter dishes and supplemented with dendroflorin 20 h after cell seeding. After 24 h treatment, cells were harvested and washed in PBS after centrifugation, then resuspended in PBS solution with 0.1% RNase and 50 μg/ml propidium iodide or culture medium containing 10 μM DCFH-DA for 30 min. DNA or ROS contents were then determined by fluorescence-activated cell sorting on a Becton-Dickinson FACScan Flow Cytometry System (Becton Dickinson, Franklin Lakes, NJ, USA). The data were analyzed by CellFIT software.

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A new diketopiperazine alkaloid isolated from an algicolous *Aspergillus flavus* strain

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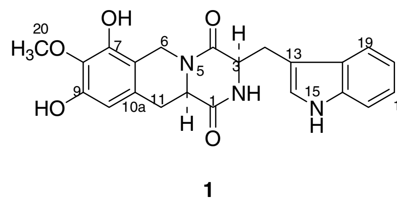
A new diketopiperazine alkaloid containing the uncommon amino acid L-7, 9-dihydroxy-8-methoxyphenylalanine (**1**), has been isolated from the algicolous *Aspergillus flavus* strain. The structure of **1** including the absolute stereochemistry was determined by spectroscopic data and chemical means. Compound **1** showed weak cytotoxicity against HL-60 cell lines with an IC_{50} value of 36.5 μg/ml.

Chemical studies of the metabolites of *Aspergillus* sp. have led to the discovery a variety of new natural products with cytotoxic and other biological activities (de Guzman et al. 1992; Cui et al. 1997), and even after investigations spanning over two decades, the genus still continues to provide metabolites with novel structures and interesting biological activities (Jian et al. 2004). In the course of searching for new antitumor compounds from marine-derived microorganisms, one new diketopiperazine (**1**) was isolated from the algicolous *Aspergillus flavus* strain, collected in Putian Pinghai, China. In this paper we describe the isolation and structure elucidation of **1**, and its cytotoxicity.



1

Compound **1** was obtained as amber amorphous solid. High-resolution ESI-MS supported the molecular formula $C_{22}H_{21}N_3O_5$ (m/z 408.1574 $[M + H]^+$, calcd. for 408.1559). The IR spectrum showed the presence of hydroxyl and amide carbonyl groups. The ^{13}C NMR and DEPT spectra displayed the characteristic structure of a diketopiperazine ring system, which include the two amide carbonyl groups at C-1 (δ_C 165.4) and C-4 (δ_C 164.0), two methine residues at C-3 (δ_C 55.7) and C-11a (δ_C 54.5) (Young et al. 2006). Careful analysis of the 1D NMR spectra indicated the presence of one disubstituded benzene ring, an N-H proton (δ_H10.82, s) and an aromatic proton (δ_H 6.91, d, J = 2.28) characteristic of a tryptophan residue (Santamaria et al. 1999), and one tetra-substituded phenylalanine residue (three of the substituted

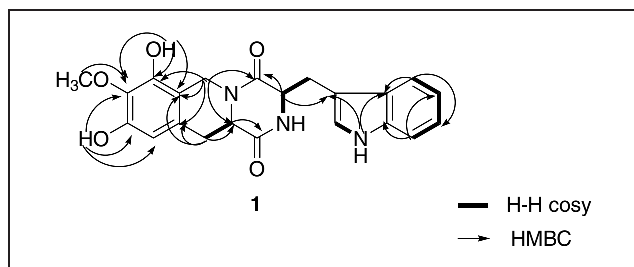


Fig: Key COSY and HMBC correlations for 1

groups were oxygenated groups and one of which was methylene group). Three oxygenated groups substituting at C-7, C-8 and C-9 were confirmed by key HMBC correlations from 7-OH to C-6a, C-7 and C-8, from 20-OCH_3 to C-8 and from 9-OH to C-8, C-9 and C-10. The correlations between H-6 and C-4, C-7, C-6a, C-10a and C-11a in HMBC spectrum implied methylene (δ_C 39.5, C-6) linking C-6a and 5-N to form a six-membered ring. Finally, HMBC correlations from H-3 to C-4 and C-13, from H-11 to C-10a and C-11a and from H-11a to C-1 unambiguously established that **1** was a diketopiperazine molecular structure derived from tryptophan and phenylalanine. The relative stereochemistry of **1** was determined by the NOE-difference experiment. When H-3 was irradiated, the signal of NH-2 was enhanced, which revealed that the two protons were on the same side of the six-membered ring. And the enhancement of NH-2 signal upon irradiation of H-11a indicated all three protons were on the same side of the diketopiperazine ring, implying that H-3 and H-11a was also *cis* arranged. The absolute configuration of C-3 of the tryptophan residue in **1** was determined by chiral HPLC analysis of the acid hydrolysate of **1**. The result L-configuration of tryptophan was assigned and thus the C-3 and C-11 were both *S*-configuration. (Shigemori et al. 1998). These data established the structure of **1** as (3*S*, 11a*S*)-3-[(1*H*-indol-3-yl)methyl]-7,9-dihydroxy-8-methoxy-2,3,11,11a-tetrahydro-6*H*-pyrazino[1,2-*b*]isoquinoline-1,4-dione.

Compound **1** was evaluated for cytotoxic activities by the MTT method (Mosmann 1983) using HL-60 and MOLT-4 cell lines, by the SRB method (Skehan et al. 1990) using A-549 and BEL-7402 cell lines. Compound **1** displayed weak cytotoxic activity against HL-60 cell lines with an IC_{50} value of 36.5 μg/ml, and showed no activity on the other cell lines at concentrations up to 40.8 μg /ml.

Experimental

1. Material and methods

Optical rotations were obtained on a JASCO P-1020 digital polarimeter. IR spectrum was taken on a Nicolet NEXUS 470 spectrophotometer in KBr disks. ^{1}H, ^{13}C NMR, DEPT spectra and 2D NMR were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as internal standard, and chemical shifts were recorded as δ values. 1D NOE spectra were obtained on a Varian INOVA-400 spectrometer. ESIMS was measured on a Q-TOF Ultima Global LC mass spectrometer. Semipreparative HPLC was performed using an ODS column (YMC-Pack ODS-A, 10 × 250 mm, 5 μm, 4 mL/min).

2. Microorganism

The *Aspergillus flavus* strain was separated from marine algae *Enteromorpha tubulosa*, collected at Putian Pinghai, China, in August, 2005. It was identified by Prof. Hong Kui and was preserved in Institute of Marine Drugs and Food, Ocean University of China (depositary number: c-f-3).

3. Fermentation

The fungus was grown under static conditions at 24 °C for 40 days in 167 × 1000-mL conical flasks containing the liquid medium (300 ml/flask) composed of glucose 1%, maltose 2%, and yeast extract 0.3%, mannitol 2% and artificial seawater after adjusting its pH to 6.5.

Table : ^{1}H and ^{13}C NMR, HMBC data for 1 (DMS0-d_6, 600 and 150 MHz, TMS, δ in ppm)

No.	δ_H (J in Hz)	δ_C	HMBC
1		165.4s	
2	8.34 (1H, s)		
3	4.26 (1H, m)	55.7d	4,13
4		164.0s	
6	Ha 4.96 (1H, d, J = 16.98) Hb 3.69 (1H, d, J = 16.98)	39.5t	4, 7, 10a, 6a, 11a 6a, 10a
6a		109.9s	
7		146.5s	
8		133.6s	
9		148.5s	
10	5.79 (1H, s)	106.3d	6a, 8, 9, 11
10a		127.7s	
11	Ha 2.29 (1H, dd, J = 15.57, 3.21) Hb 0.91 (1H, t, J = 13.98)	32.1t	6a, 10a 10a, 11a
11a	3.78 (1H, dd, J = 12.39, 3.21)	54.5d	1
12	Ha 3.27 (1H, dd, J = 14.64, 3.72) Hb 3.04 (1H, dd, J = 14.64, 4.35)	29.8t	13, 19a 3, 4, 13
13		108.1s	
14	6.91 (1H, d, J = 2.28)	124.2d	13, 15a, 19a
15	10.82 (1H, s)		13, 15a, 19a
15a		135.6s	
16	7.50 (1H, d, J = 7.74)	118.7d	15a, 18
17	6.82 (1H, td, J = 7.32, 0.84)	118.2d	19a
18	6.93 (1H, td, J = 7.32, 0.96)	120.7d	
19	7.23 (1H, d, J = 8.22)	110.9d	17, 19a
19a		127.7s	
20	3.62 (3H, s)	59.8q	8
7-OH	8.86 (1H, s)		6a, 7, 8
9-OH	8.93 (1H, s)		8, 9, 10

4. Extraction and isolation

The fermented whole broth (50 L) was filtered through cheesecloth to separate into supernatant and mycelia. The supernatant was extracted with ethyl acetate, while the mycelia were extracted with 70% acetone-aqueous. The acetone solution was concentrated under reduced pressure to afford an aqueous solution, which was extracted with ethyl acetate. Both ethyl acetate solutions were combined and concentrated under reduced pressure to give a crude extract (77.7 g). The crude extract was separated into eight fractions on a silica gel column using a step gradient elution of $CHCl_3$/MeOH. Fraction 7 was chromatographed on a Sephadex LH-20 column with 50% $CHCl_3$/MeOH to give six fractions, the fourth fraction was purified by semipreparative HPLC (40% MeOH/H_2O) to give compound **1** (26 mg).
(3*S*, 11a*S*)-3-[(1*H*-Indol-3-yl)methyl]-7, 9-dihydroxy-8-methoxy-2,3,11,11a-tetrahydro-6*H*-pyrazino[1,2-*b*] isoquinoline-1,4-dione (**1**): Amber amorphous solid; $[\alpha]_D^{20}-228.7$ (c 0.100, MeOH); HRESIMS *m/z* 408.1574 for $[M+H]^+$ (calcd for $C_{22}H_{21}N_3O_5$, 408.1559); IR (KBr) υ_{max} 3350, 2928, 1658, 1452, 1343, 1246, 1079 cm^{-1}; UV (λ_{max}, MeOH): 217 (4.30), 269 (3.99); ^1H and ^{13}C NMR (DMS0-d_6, 600 MHz and 150 MHz) see Table 1.

5. Amino acid analysis of hydrolysate of 1

Compound **1** (1 mg) was dissolved in 6N HCl (1.5 ml) and heated to 110 °C for 24 h in a sealed tube. The excess HCl was removed under vacuum. The dry hydrolysate was dissolved in H_2O for chiral HPLC analysis. The chiral HPLC analysis was carried out using a CROWNPAK® CR (+) column [Column Size, 4 × 150 mm; flow rate, 1.2 mL/min; eluent, $HClO_4$, PH 2; detection, UV at 210 nm; column temperature, 30 °C]. Retention times of standard L- and D-Try, hydrolysate of **1** were 26.11, 21.82 and 26.12 min, respectively.

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The history of cholinesterase inhibitors: who was Moschnin(e)?

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The synthesis of the first organophosphate cholinesterase inhibitor (tetraethyl pyrophosphate, TEPP) is often credited to the French organic chemist Philippe de Clermont, working in the laboratories of Adolphe Wurtz in Paris. The two de Clermont's publications dealing with TEPP clearly state however that the first synthesis of TEPP was achieved by another student of Wurtz, named Moschnine. While de Clermont is well known, nobody really knows who Moschnine was. This brief communication attempts to give an overview about the life and achievements of the Russian chemist Wladimir Petrovich Moshnin from Moscow.

Most people interested in organophosphorus cholinesterase inhibitors and their history will be familiar with Bo Holmstedt's chapter in Koelle's Textbook "Cholinesterases and Anticholinesterase Agents" published almost half a century ago (Holmstedt, 1963). It contains a superb account of the synthesis of the first organophosphate cholinesterase inhibitor (tetraethyl pyrophosphate, TEPP) by the French organic chemist Philippe de Clermont (1831–1921), working in the laboratories of Adolphe Wurtz (1817–1884) in Paris. Paragraphs from the two de Clermont's publications dealing with TEPP are reproduced by Holmstedt, including de Clermont's statement that TEPP was actually synthesized earlier by another student of Wurtz, named Moschnine (de Clermont, 1853, 1855). Holmstedt concludes his remarks dealing with the very early days of organophosphate chemistry by noting that "*Nobody knows who Moschnine was*."
Confronted with this blunt statement in my early professional years, I refused to accept the reality and spent time off and on unsuccessfully searching for Mochnine, Moshnin, or Moschnin.
Some twenty years of sporadic and futile search efforts came to an end through my contact with a young Portuguese researcher, Ana Carneiro, author of a Ph.D. thesis entitled "The Research School of Chemistry of Adolphe Wurtz," who not only sent me a copy of her work, but also patiently answered my questions. Through her, I obtained two hints that allowed me to identify the elusive Moschnin (Carneiro 1992).
First, she pointed the way to Charles Friedel's introductory remarks to Wurtz's book *La Theorie Atomique* (1886).