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Chrysogenamide A from an Endophytic Fungus Associated with *Cistanche deserticola* and Its Neuroprotective Effect on SH-SY5Y Cells

Zhenjian Lin, Jiangni Wen, Tianjiao Zhu, Yuchun Fang, Qianqun Gu, Weiming Zhu

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Abstract Chrysogenamide A (**1**), a new member of the macfortine group of alkaloids, along with four known compounds (**2**~**5**) were identified from *Penicillium chrysogenum* No. 005, an endophytic fungus associated with *Cistanche deserticola* Y. C. Ma. The new structure was elucidated on the basis of comprehensive spectral analysis. **1** exhibited a neurocyte protection effect against oxidative stress-induced cell death in SH-SY5Y cells.

Keywords chrysogenamide A; endophyte; *Penicillium chrysogenum*; *Cistanche deserticola*; neurocyte protection; marcfortine alkaloids

Introduction

Cistanche deserticola (Y. C. Ma) is a parasitic plant native to the northwest of China. The stem of this plant is an important traditional Chinese medicine and is used for the treatment of kidney deficiency and neurasthenia ("The Chinese Medicine Dictionary", 1977). Phenylthanoid glycosides from *C. deserticola* have been reported to have activities in preventing and treating cranial nerve diseases and scavenging free radicals [1, 2]. Based on the fact that endophytic fungi can produce new medicinal components and the same/similar ones as those from the host plants, searching for new active compounds from endophytic fungi of important medicinal plants shows value for the development of new medicinal resources. In the course of our search for new neuroprotective compounds from endophytic fungi from *C. deserticola* collected from Inner Mongolia in northwest China, three active strains were screened using SH-SY5Y cells. Among them, the culture broth of a fungus No. 005, authenticated as *Penicillium chrysogenum*, showed a significant neurocyte protection effect against oxidative stress-induced cell death in SH-SY5Y cells. Studies on the active constituents of this strain led to the isolation of chrysogenamide A (**1**) (Fig. 1) through a bioassay-guided isolation procedure. In addition, four known compounds were isolated and identified as circumdatin G (**2**) [3], 2-[(2-hydroxypropionyl) amino] benzamide (**3**) [4], 2′,3′-dihydrosorbicillin (**4**) [5] and (9*Z*,12*Z*)-2,3-dihydroxypropyl octadeca-9,12-dienoate (**5**) [6], by comparison of their spectroscopic data with literature data. Here, we report on the isolation, identification and neuroprotective effect of chrysogenamide A.

Materials and Methods

Fungal Material

A sample of *Penicillium chrysogenum* No. 005 was isolated from the root of *C. deserticola* collected from Inner Mongolia in northwest China. On potato dextrose agar the macroscopic appearance was of spreading, smooth, velvety, blue-green colonies sometimes overgrown with light hyphae; the surface of the colonies often showed numerous light yellow droplets; reverse yellow. A GenBank search with the 18S ribosomal DNA gene sequence of No. 005 (1676 nucleotides; Gen-Bank accession number

Q. Gu, W. Zhu (Corresponding author)**, Z. Lin, J. Wen, T. Zhu, Y. Fang:** Key Laboratory of Marine Drugs, Chinese Ministry of Education; School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, P.R. China,
E-mail: guqianq@ouc.edu.cn and weimingzhu@ouc.edu.cn

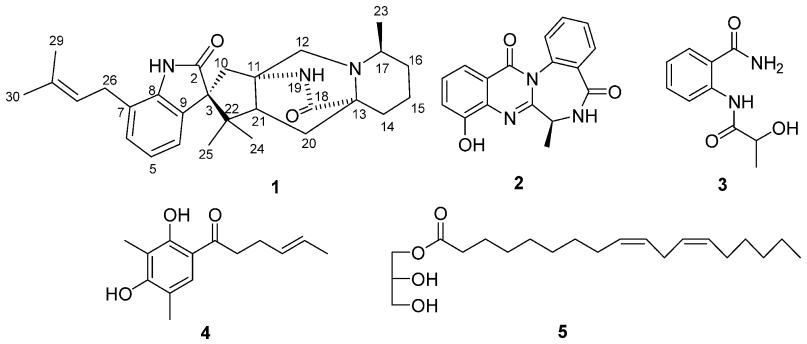

Fig. 1 Structures of chrysogenamide A (**1**), circumdatin G (**2**), benzamide (**3**), 2′,3′-dihydrosorbicillin (**4**), (9*Z*,12*Z*)-2,3-dihydroxypropyloctadeca-9,12-dienoate (**5**).

EU203859) was carried out. The results indicate a species of *Penicillium chrysogenum* as the closest match. On the basis of its macroscopic appearance and 18S rDNA gene sequence, No.005 is a strain of *Penicillium chrysogenum*.

Fermentation and Extraction

The fungus was grown under static conditions at 24°C for 30 days in 35 1000-ml conical flasks containing a liquid medium (300 ml/flask) composed of glucose (10 g/liter), maltose (20 g/liter), mannitol (20 g/liter), monosodium glutamate (10 g/liter), KH_2PO_4 (0.5 g/liter), $MgSO_4 \cdot 7H_2O$ (0.3 g/liter), corn steep liquor (1.0 g/liter) and yeast extract (3.0 g/liter) and seawater after adjusting its pH to 7.0. The fermented whole broth (10.5 liters) was extracted three times with EtOAc to give an EtOAc soln, which was concentrated under reduced pressure to give a crude extract (9.5 g).

Purification

The crude extract (9.5 g) was separated into 15 fractions on a silica gel column using a step gradient elution of petroleum ether/Me_2CO. The fraction 2, eluted with 8 : 2 petroleum ether/Me_2CO, was recrystallized from MeOH, yielding **3** as colorless needles (8.0 mg). The fraction 3 (19.0 mg), eluted with 7 : 3 petroleum ether/Me_2CO, was further separated by reversed-phase column (RP18, 40～60 μm, Merck; MeOH - H_2O=3 : 2→4 : 1→MeOH) to afford fr. 3～7 (8.0 mg), which was purified by extensive PHPLC (60% MeOH containing 0.1% TFA, 4.0 ml/minute) to give **4** (2.0 mg). The fraction 4 (26.0 mg), eluted with 6 : 4 petroleum ether/Me_2CO, was further separated by reversed-phase column (RP18, 40～60 μm, Merck; MeOH - H_2O=3 : 2→4 : 1→MeOH) to afford fr. 4-3 (5.4 mg) and fr. 4-9 (7.6 mg). Then, the fr. 4-3 was separated by extensive PHPLC [YMC-pack ODS (A), 10×250 mm, 5 μm, 4.0 ml/minute] (60% MeOH, 4.0 ml/minute) to yield **2** (1.0 mg), and the fr. 4-9 was separated by extensive PHPLC (80% MeOH, 4.0 ml/minute) to yield **1** (4.0 mg) and **5** (2.6 mg).

1: obtained as pale yellow solid; $[\alpha]_D^{25}$ +24.5° (*c* 0.155, MeOH); UV (MeOH) λ_{max} 208, 251 nm; CD (MeOH) (see Fig. 4); IR (KBr) ν_{max} 3433, 3200, 3076, 2928, 2862, 1677, 1440, 1370, 1324, 1188, 1029 cm^{-1}; ^1H- and ^{13}C-NMR (see Table 1); HRESI-MS *m*/*z* 448.2951 $[M+H]^+$ (calcd for $C_{28}H_{38}N_3O_2$, 448.2964).

DPPH Radical Scavenging Effect

A MeOH soln (160 μl) of varying sample concentrations (1.0 mg/ml～0.0001 mg/ml) was added to 40 μl DPPH (1,1-diphenyl-2-picrylhydrazyl; Sigma) MeOH soln (1.5×10^{-4} M). After mixing gently and leaving for 30 minutes at room temperature, the optical density was measured at 520 nm using a spectrophotometer. The antioxidant activity of each sample was expressed in terms of IC_{50} (μg/ml or μM required to inhibit DPPH radical formation by 50%) and calculated from the log-dose inhibition curve.

Cell Culture and Drug Treatment

Human neuroblastoma SH-SY5Y cells were cultured in DMEM medium and maintained at 37°C in a humidified atmosphere with 5.0% CO_2. These cell suspensions (180 μl) at a density of 5×10^4 cell ml^{-1} were plated in 96-well microtiter plates and incubated for 24 hours at the above condition. Chrysogenamide A solution (2.0 μl in DMSO) at different concentrations was added to each well except the control and model groups and further incubated for 1 hour in the same conditions. H_2O_2 (Sigma Chemical Co., MO, USA) was added to induce apoptosis in SH-SY5Y cells at a final concentration of 200 μM for 24 hours. Cell viability was determined using an MTT assay. MTT dissolved in phosphate buffered saline was added at the end of incubation to a final concentration of 5.0 mg/ml (PBS), and then incubated at 37°C for 4 hours. Discarding the supernatant, the resultant formazan product was dissolved

Table 1 ^{1}H- and ^{13}C-NMR data (600 and 150 MHz) of chrysogenamide A (**1**) in DMSO-d_6

No.	^{1}H (J=Hz)	^{13}C
1-NH	10.36 s	—
2	—	183.1, qC
3	—	62.3, qC
4	7.18 d (7.3)	123.6, CH
5	6.90 dd (7.3, 7.3)	120.8, CH
6	6.94 d (7.3)	127.7, CH
7	—	122.1, qC
8	—	140.4, qC
9	—	129.9, qC
10a	2.20 m[a]	39.9, CH_2
10b	2.09 m[a]	
11	—	60.4 qC
12a	3.32 d (8.7)	64.9, CH_2
12b	2.22 m[a]	
13	—	58.7, qC
14a	1.44 m[a]	20.7, CH_2
14b	1.15 m[a]	
15a	2.08 m	31.8, CH_2
15b	10.36 s	
16a	1.52 m	34.3, CH_2
16b	1.08 m	
17	2.02 m	58.7, CH
18	—	172.3, qC
19-NH	8.29 s	
20a	1.63 dd (10.1, 13.2)	35.2, CH_2
20b	1.37 dd (7.8, 13.2)	
21	2.89 dd (9.1, 9.2)	46.1, CH
22	—	46.5, qC
23	0.98 d (6.0)	21.5, CH_3
24	0.62 s	22.7, CH_3
25	0.87 s	20.1, CH_3
26	3.23 m	28.4, CH_2
27	5.27 dd (7.3, 7.3)	121.9, CH
28	—	132.3, qC
29	1.70 s	25.5, CH_3
30	1.67 s	17.7, CH_3

[a] Signals overlapping. qC; quarternaly carbon.

with 200 μl DMSO and detected at 570 nm.

Results and Discussion

1 was obtained as pale yellow solid. The molecular formula was determined as $C_{28}H_{37}N_3O_2$ by HRESI-MS (observed $[M+H]^+$ at m/z 448.2951, calcd $[M+H]^+$ $C_{28}H_{38}N_3O_2$, 448.2964). The IR spectrum suggested the presence of NH (3433 and 3200 cm^{-1}) and carbonyl group(s) (1677 cm^{-1}). The ^{1}H-NMR (Table 1) spectrum recorded in DMSO-d_6 revealed three methyl groups (δ_H 0.62, 0.87, 0.98), and two exchangeable-proton signals (δ_H 10.36 and 8.29). Moreover, three *ortho* coupled aromatic signals (δ_H 6.90 dd, J=7.3, 7.3 Hz, 6.94 d, J=7.3 Hz and 7.18 J=7.3 Hz) indicated a 1,2,3-trisubstituted benzene ring. The methylene protons (δ_H 3.23) coupled with a olefinic proton (δ_H 5.27), along with two vinylic methyls (δ_H 1.67 and 1.70), indicated the presence of a 3-methyl-2-butenyl group in **1**. The one-bond connectivities of these protons and another 14 methine and or methylene protons were established by a HMQC experiment. The ^{13}C-NMR and DEPT (Table 1) spectra, which disclosed 28 signals, also confirmed the presence of these two fragments [trisubstituted benzene ring (δ_C 123.6 CH, 120.8 CH, 127.7 CH, 122.1 qC and 140.4 qC) and a 3-methyl-2-butenyl (δ_C 28 CH_2, 121.9 CH, 132.3 qC, 25.5 CH_3 and 17.7 CH_3)]; in addition, two carbonyls (δ_C 183.1 and 172.3), four sp^3 quaternary carbons (δ_C 46.5, 58.7, 60.4, and 62.3), two sp^3 methines (δ_C 46.1 and 58.7), and six sp^3 methylenes (δ_C 20.7, 31.8, 34.3, 35.2, 39.9 and 64.9) were also observed. Since 6 out 12 unsaturations were accounted for, **1** was inferred to contain six rings. Further evaluation of these spectral data, along with ^{1}H-^{1}H COSY and HMBC results, revealed that **1** is closely related to the marcfortine group of fungal metabolites [7]. Interpretation of HMBC correlations (Fig. 2) indicated the presence of partial structure A (HMBC correlations 1-NH to C-8 and C-9; H-6 to C-26; H-5 to C-7 and C-9; H-26 to C-28 and C-8 and H-27 to C-29 and C-30) and B (HMBC correlations H-10 to C-3, C-22, C-21, C-11, C-12; H-20 to C-13, C-18, C-22, C-11 and 19-NH to C-13, C-10), and C, a six membered G ring [8] with a methylated carbon at C-17 (HMBC correlations H-23 to C-17, C-16). The connectivity of substructure A to B through C-3 was supported by the key HMBC correlations from H-4 and 1-NH to C-3; H-10 to C-2 and C-9. The connection between structure B and C with a nitrogen atom was deduced by the key HMBC correlations from H-12 to C-17 and C-13 and comparing their corresponding chemical shifts of C-12 and C-17 (δ_C 64.9 and 58.7). The above analysis indicated that **1** had a similar structure to marcfortine **A** except for the differences of the side chain at benzene ring and a methyl at C-17 of the six membered G ring. So the gross structure of **1** was assigned as shown in Fig. 1.

The relative stereochemistry of **1** was elucidated on the basis of NOESY data (Fig. 3). Previous studies have revealed that the 2-oxindole alkaloid type compounds adopted a conformation in which the central five-membered ring is orthogonal to the plane of the oxindole subunit [9].

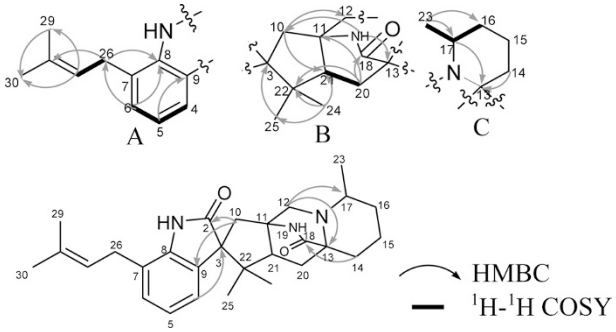

Fig. 2 Key HMBC and ^{1}H-^{1}H COSY correlations of **1**.

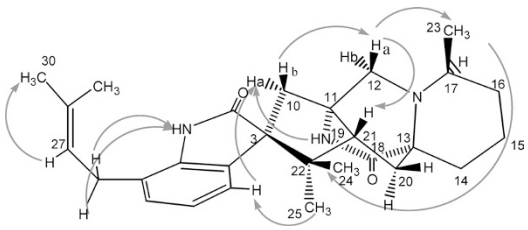

Fig. 3 NOESYs of **1**.

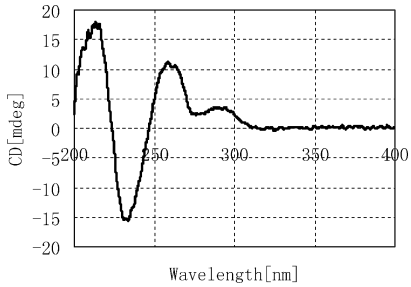

Fig. 4 CD spectrum of **1**.

NOESY correlations between H-4 and H-10a, H-25 and between 19-NH and H-10a indicated that they were both on the face of the cyclopentanoid ring that orients them toward H-4, fixing the relative stereochemistry at C-3 as shown. NOESY interactions between H-21, H-12a, H-10b, H-23 and H-24 placed the corresponding substituents together on the frontal face of the cyclopentanoid ring. The relative stereochemistry proposed for **1** is in agreement with the relevant features of the relative stereochemistry of marcfortine A [7]. The Cotton effect at 200～250 nm arises from an n-π* transition of the diketopiperazine amide bonds, which is diagnostic for the bicyclo-[2.2.2] diazaoctane diketopiperazine core [10, 11]. The CD spectrum of **1** (Fig. 4) correlated to relevant regions of that of (+)-brevianamide A [11]. So the absolute stereochemistry of **1** should be 3*S*, 11*S*, 13*S*, 17*S*, 21*R* and was given the name of chrysogenamide A.

Preliminary evaluation of the protective effect of chrysogenamide A on neurocytes was evaluated using oxidative stress-induced cell death in SH-SY5Y cells by an MTT assay. The free radical scavenging activity of chrysogenamide A was first examined using DPPH as a free radical donor. In contrast to vitamin C (IC_{50}: 29.0 μM), chrysogenamide A did not show any ability (IC_{50} >100 μM) to scavenge DPPH free radical at a concentration up to 100 μM. Meanwhile we tested whether chrysogenamide A protected cells against oxidative stress-induced cell death in SH-SY5Y human neuroblastoma cells. The oxidative stress by hydrogen peroxide resulted in a decrease in the cell viability by 43% as compared with control group. Chrysogenamide A inhibited cell death induced by hydrogen peroxide by improving cells viability by 59.6% at concentration of 1×10^{-4} μM. These data suggested that chrysogenamide A showed a neurocyte protection effect against oxidative stress-induced cell death, not through its antioxidant activity.

Indole alkaloids possessing an unusual bicyclo [2.2.2] diazaoctane ring system have been isolated from different genera of fungi such as *Aspergillus* [12, 9, 13], *Penicillium* [14～16] and *Malbranchea* [17]. **1** was a new member of this group of compounds, it possessed a skeleton expected to be an intermediate in the biosynthesis of the citrinadins A and B reported as a novel class of pentacyclic spiroindolinone alkaloids in 2005 [18]. From the structural point of view, it is important to point out that **1** is a unique structure with methylation at position C-17 and a 2-oxindole moiety possessing an isoprene unit at C-7. Some of these indol alkaloids isolated to date exhibit a potent antiparasitic activity [19], inhibition of human tumor cell [13] and cytotoxicity [10]. To the best of our knowledge, it is the first report of neuroprotection by this type of alkaloids.

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