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Trichodermamide A and Aspergillazine A, Two Cytotoxic Modified Dipeptides from a Marine-Derived Fungus *Spicaria elegans*

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Two known modified dipeptides, trichodermamide A (**1**) and aspergillazine A (**2**), were isolated from an ethyl acetate extract of the metabolite of a marine-derived fungus *Spicaria elegans*, and were found to have a weak cytotoxic effect on three cancer cell lines P388, A-549, and HL-60 agreed. To our knowledge, this is the first report on the isolation of compounds **1** and **2** from the fungus *Spicaria elegans* and their cytotoxic effect.

Key words: Marine-derived fungus, *Spicaria elegans*, Trichodermamide A (penicillazine), Aspergillazine A, Modified dipeptides, Cytotoxic activity

INTRODUCTION

Natural products with novel structures and distinct biological activities have been attracting much attention in the development of new medicine, and several of these products have been secondary metabolites of marine-derived microbes (Bugni and Irland, 2004). In to the ongoing search for anticancer compounds, more than 2000 microbial strains isolated from sediment samples collected from China's Jiaozhou Bay were screened using mammalian tsFT210 cells (Cui *et al.*, 2001a, 2001b). Among them, a fungal strain identified as *Spicaria elegans* exhibited cytotoxic activity. The active components of this strain were investigated by a bioassay-guided isolation procedure, which resulted in the isolation of three new cytochalasins (Z_7, Z_8, Z_9) and two known cytochalasins E and K (Liu *et al.*, 2005). Further investigation of *Spicaria elegans* led to the isolation of two known modified dipeptides, trichodermamide A (**1**) and aspergillazine A (**2**) from the mass re-culture of this strain in the same media. In this paper we report on the isolation of these compounds, their structure, and cytotoxic activity against P388, A-549, and HL-60 cell lines.

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MATERIALS AND METHODS

General experimental procedure

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 a Beckmen DU® 640 spectrophotometer. IR spectra were recorded on a NICOLET NEXUS 470 spectrophotometer in KBr discs. ^1H-, ^{13}C-NMR, and DEPT spectra and 2D-NMR were recorded on a JEOL Eclips-600 spectrometer using TMS as the internal standard, and chemical shifts were recorded as δ values. EI-MS was carried out on a Finnigan-MAT 95 mass spectrometer, and 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].

Fungal material

The fungus, *Spicaria elegans*, was isolated from the marine sediments collected in Jiaozhou Bay, China. It was preserved in the China Center for Type Culture Collection (patent depositary number: KLA03 CCTCC M 205049). Working stocks were prepared on Potato Dextrose agar slants stored at 4°C.

Fermentation and extraction

The fungus was grown under static conditions at 24°C

for 25 days in eighty 1000 mL conical flasks containing the liquid medium (300 mL/flask) which was composed of glucose (20 g/L), peptone (5 g/L), malt extract (3 g/L) and yeast extract (3 g/L) and sea-water with an adjusted pH of 7.0. The fermented whole broth (15 liters) was filtered through cheese cloth to separate the supernatant from the mycelia. The supernatant was concentrated under reduced pressure to about a quarter of the original volume and then extracted three times with ethyl acetate to produce an ethyl acetate solution, while the mycelia were extracted three times with acetone. The acetone solution was concentrated under reduced pressure to produce an aqueous solution. The aqueous solution was extracted three times with ethyl acetate which yielded another ethyl acetate solution. Both ethyl acetate solutions were combined and concentrated under reduced pressure to produce a crude extract (23.0 g).

Purification

The crude extract (23.0 g), showing cytotoxity against tsFT210 cells, was separated into 15 fractions on a silica gel column using a step gradient elution of petroleum ether:acetone. The isolation of compounds **1** and **2** was achieved by a bioassay-guided fractionation procedure. Briefly, bioactive fraction 13, eluted with petroleum ether: acetone 5:5 (1.4 g), was purified into 6 subfractions by another silica gel column using an isocratic elution of $CHCl_3$:MeOH 95:5. Among these 6 subfractions the active subfraction 13-2 was recrystallized from acetone, yielding compound **1** as colorless needles (11 mg). Subfraction 13-3 was further purified by preparative HPLC (60% MeOH) yielding compound **2** (7 mg, t_R 7 min) as a yellow amorphous powder.

Trichodermamide A (1)

Colorless needle; m.p. 258-260°C; $[\alpha]_D^{20}$ +88.6° (*c* 0.07, MeOH); UV (MeOH) λ_{max} nm (log ε) 266 (4.13), 344 (4.76); IR (KBr) ν_{max} 3482, 2919, 1725, 1683, 1606, 1526, 1379, 1086, 1008, 903 cm^{-1}; ESI-MS *m/z* 433 $[M + H]^+$; EI-MS *m/z* 432 $[M]^+$; HRESI-MS *m/z* 433.1272 $[M + H]^+$ (calcd for $C_{20}H_{21}N_2O_9$, 433.1247); ^{1}H- and ^{13}C-NMR (see Table I).

Aspergillazine A (2)

Yellow amorphous powder; $[\alpha]_D^{20}$ -366.4° (*c* 0.115, MeOH); UV (MeOH) λ_{max} nm (log ε) 270 (3.95), 338 (4.29); IR (KBr) ν_{max} 3443, 2919, 1719, 1686, 1653, 1420, 1102, 1046, 970 cm^{-1}; ESI-MS *m/z* 449 $[M+H]^+$; EI-MS *m/z* 448 $[M]^+$; HREI-MS *m/z* 448.0925 $[M]^+$ (calcd for $C_{20}H_{20}SN_2O_8$, 448.0940); ^{1}H- and ^{13}C-NMR (see Table II).

In vitro cytotoxicity assays

Active fractions were assayed using the MTT method

Table I. ^{1}H- and ^{13}C-NMR (600 and 150 MHz) data for trichodermamide A (**1**) in acetone- d_5

No.	δc	δ_H (*J* in Hz)	COSY	HMBC
1	162.1 s			
2	151.1 s			
3	29.5 t	Ha 2.71 (dd, 19.4, 2.2), Hb 2.26 (d, 19.4)	H-3b, H-9, H-3a	C-1, C-2, C-4, C-9, C-2, C-4, C-5
4	68.8 s	2.83 (br s), 4-OH	H-3a, H-3b	
5	74.7 d	4.51 (br s), 4.46 (br s), 5-OH	H-6, H-7, H-8	C-3, C-4, C-6, C-9, C-5, C-6
6	130.7 d	5.58 (ddd, 10.2, 2.2, 2.2)	H-5, H-7, H-8	C-8
7	128.8 d	5.55 (ddd, 10.2, 2.2, 2.2)	H-5, H-6, H-8	C-5, C-9
8	67.8 d	4.23 (br s), 4.67 (d, 5.5), 8-OH	H-5, H-6, H-7, OH-8, H-8	C-9, C-7, C-8, C-9
9	85.1 d	4.14 (dd, 8.0, 2.2)	H-3a, H-8	C-3, C-5, C-8
1'	158.9 s			
2'	122.3 s			
3'	123.7 d	8.58 (s)		C-1', C-2', C-9'
4'	114.9 s			
5'	123.5 d	7.41 (d, 8.8)	H-6'	C-3', C-4', C-6', C-7', C-8', C-9'
6'	110.7 d	7.12 (d, 8.8)	H-5'	C-4', C-7', C-8', C-9'
7'	155.1 s			
8'	136.8 s			
9'	144.9 s			
7'-OMe	56.7 q	3.95 (s)		C-7'
8'-OMe	61.3 q	3.90 (s)		C-8'
CONH		9.27 (s)		

Table II. ^{1}H and ^{13}C NMR (600 and 150 MHz) data for aspergillazine A (**2**) in acetone- d_5

No.	δc	δ_H (*J* in Hz)	COSY	NOE's	HMBC
1	160.7 s				
2	76.5 s				
3	50.3 t	Ha 3.12 (d, 11.7), Hb 2.40 (d, 11.7)	H-3b, H-3a	H-5, H-9	C-4.C-5, C-9, C-1, C-4, C-5, C-9
4	76.2 s	5.22 (br s), 4-OH		H-5, 8-OH, H-9	C-3, C-4, C-9
5	47.3 d	4.15 (d, 4.8)	H-6, H-9	H-3a, 4-OH	C-4, C-6, C-9
6	126.0 d	5.94 (dd, 11.1, 4.8)	H-5, H-7		C-4, C-8
7	127.9 d	6.05 (ddd, 11.1, 5.2, 1.1)	H-6, H-8		C-5
8	65.0 d	4.31 (br s), 4.61 (d, 5,2), 8-OH	H-7, H-9, OH-8 H-8	4-OH, H-9	C-7, C-9
9	80.1 d	4.21 (d, 1.1)	H-5, H-8	H-3b, 4-OH, H-8	C-3, C-5, C-7, C-8
1'	157.0 s				
2'	122.4 s				
3'	115.7 d	6.88 (s)			C-1', C-5', C-9'
4'	114.7 s				
5'	128.3 d	7.08 (d, 8.8)	H-6'		C-3', C-7', C-8', C-9'
6'	105.7 d	6.67 (d, 8.8)	H-5'		C-4', C-7', C-8' C-9'
7'	154.9 s				
8'	137.3 s				
9'	148.0 s				
7'-OMe	56.3 q	3.89 (s)			C-7'
8'-OMe	60.9 q	3.81 (s)			C-8'
CONH		9.27 (s)			
-O-NH		9.90 (s)			

with mouse temperature-sensitive p34^{cdc2} mutant cell line tsFT210. Cytotoxic activity was evaluated by the MTT method (Mosmann *et al.*, 1983) using P388, A-549, and HL-60 cell lines. The cell lines were grown in RPMI-1640 supplemented with 10% FBS in a humidified atmosphere of 5% CO_2 and 95% air at 37°C (tsFT210 cell line at 32°C). Next, 200 μL of those cell suspensions at a density of 5×10^4 cell mL^{-1} was plated in 96 well microtiter plates and incubated for 24 h in the same condition described above. Then 2 μL of the test compound solutions (in DMSO) at different concentrations was added to each well and incubated further for 72 h in the same condition. 20 μL of the MTT solution (5 mg/mL in IPMI-1640 medium) was added to each well and incubated for 4 h. 150 μL of an old medium containing MTT was then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a SPECTRA MAX PLUS plate reader at 540 nm.

RESULTS AND DISCUSSION

The two known compounds trichodermamide A (**1**) and aspergillazine A (**2**) were isolated from an ethyl acetate extract of *Spicaria elegan* by bioassay-guided fractionation and showed cytotoxicity against tsFT210 cell line. Ed. Note: confirm wording. Compounds **1** and **2** were identified by analysis of their NMR data as well as by a comparison of their physical and spectral data with those of literature (Lin *et al.*, 2000; Caro *et al.*, 2003; Capon *et al.*, 2005). To our knowledge, this is the first isolation of compounds **1** and **2** from this fungus.

Compound **1** was isolated and presented as colorless needles and had a melting point of 258-260 C. It was analyzed by EI-MS (*m/z* 432 [M]$^+$), ESI-MS (*m/z* 433 [M + H]$^+$), HRESI-MS (*m/z* 433.1217 [M + H]$^+$, calcd 433.1247), and NMR methods for $C_{20}H_{20}N_2O_9$, a formula requiring 12 degrees of unsaturation. The ^{1}H-NMR spectrum of compound **1** displayed three aromatic protons, one of which appeared at a low field (δ_H 8.58), two olefinic protons, three methine protons between δ_H 4.1 and 4.7, two methoxy groups, and one methylene proton. The spectrum also illustrated four exchangeable protons, including three that correspond to hydroxyl protons (δ_H 2.83, δ_H 4.46, and δ_H 4.67) and one at a low field (δ_H 9.27), assigned to be an NH proton. The ^{13}C-NMR and DEPT data showed the presence of two carbonyl carbons, 11 aromatic and olefinic sp^2 carbons, three oxygenated CH, two OCH_3, one oxygenated quaternary sp^3 carbon, and one methylene carbon. Overall, these 1D NMR data suggested that compound **1** was composed of two modified amino acids, one of which

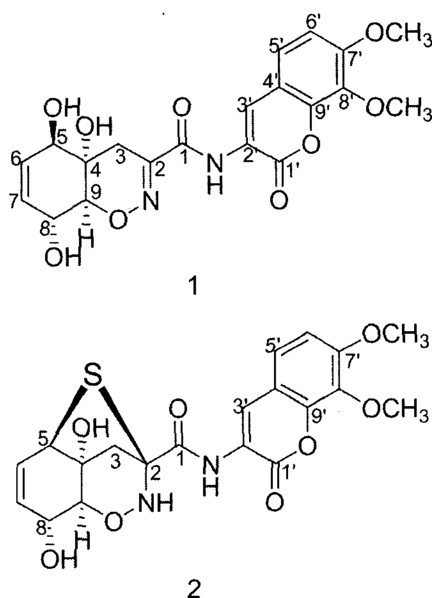

Fig. 1. Structures of trichodermamide A (**1**) and aspergillazine A (**2**)

was aromatic, arranged in a tetracyclic ring system. A series of HMBC correlations such as between H-3' with C-1', 2', 9' among others shown in Table I led to the assignment of a dimethoxylated benzopyran ring. The second part of the molecule was also be assigned by the COSY and HMBC correlations (see Table I). In addition, the prominent peaks at EI-MS *m/z* 206 [M - $C_9H_{11}N_2O_5$ + H]$^+$ and 221 [M - $C_9H_{10}NO_5$ + H]$^+$ confirmed the presence of the dimethoxylated benzopyran ring moiety and the amide group, as a result it received a planar structure. Ed. Note: confirm wording. The relative and absolute stereochemistry was determined by comparing its ^{1}H- and ^{13}C-NMR chemical shifts, and coupling constants and positive optional rotation data with data found in literature (Caro *et al.*, 2003). Consequently, compound **1** was identified as trichodermamide A (penicillazine).

Compound **2** was isolated and presented as a yellow powder. It was analyzed by EI-MS (*m/z* 448 [M]$^+$), ESI-MS (*m/z* 449 [M + H]$^+$), HREI-MS (*m/z* 448.0925 [M]$^+$, calcd 448.0940), and NMR methods for $C_{20}H_{20}N_2O_8S$, a formula also requiring 12 degrees of unsaturation. Detailed analysis and comparison of the ^{1}H- and ^{13}C-NMR data with those of compound **1** revealed that compound **2** was a cyclized analogue of compound **1** in which an additional heterocyclic ring had assembled through intramolecular nucleophilic addition at C-2 with the dimethoxylated benzopyran ring being identical. This conclusion was confirmed by the COSY and HMBC experiments (see Table II). In addition, the prominent peaks at *m/z* 205.0494 [M - $C_9H_{11}N_2O_4S$]$^+$ (calcd 205.0501) and 221.0682 [M - $C_9H_{10}NO_4S$ + H]$^+$ (calcd 221.0688) in its HREI-MS, confirmed the presence of the dimethoxylated benzopyran ring moiety and the amide group. The relative stereochemistry was determined by NOE and NOESY correlations (see Table II), which was consistent with that found in literature (Capon *et al.*, 2005). The absolute stereochemistry of compound **2** was tentatively assigned as shown on biogenetic grounds by comparing it to the co-metabolite compound **1**.

Trichodermamide A (**1**) was first reported from a marine-derived *Penicillium* sp. called penicillazine (Lin *et al.*, 2000). More recently, Fenical *et al.* reported the isolation and identification of trichodermamide A (**1**) and B from a marine-derived fungus *Trichoderma virens*, and noted that trichodermamide A and penicillazine "...may be identical", implying that the original structure assigned to penicillazine was incorrect (the only difference between the two reported structures is the translocation of the ester and amide bonds, Caro *et al.*, 2003). Recently, Robert J. Capon *et al.* reported trichodermamide A (**1**) again and first reported aspergillazine A (**2**) (Capon *et al.*, 2005). Aspergillazine A (**2**) was a cyclized analogue of compound **1** in which an additional heterocyclic ring had assembled through intramolecular nucleophilic addition at C-2. This research further proved the presence of an aromatic amino acid ester ring moiety in this kind of modified dipeptides by analysis of (HR)EI-MS data of these two molecules.

Cytotoxic effects of compounds **1** and **2** were first evaluated on P388, A-549 and HL-60 cell lines by MTT method (Mosmann *et al.*, 1983). Compounds **1** and **2** both displayed weak cytotoxicity against HL-60 cell line with IC_{50} values at 89 μM and 84 μM respectively. Furthermore, neither of them reached an IC_{50} value of 100 μM against p388 and A-549 cell lines. Ed. Note: confirm wording. The above result showed negligible cytotoxic effect of S-substituted heterocyclic ring. Other bioactivities of this kind of compounds need further investigation.

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