ORIGINAL ARTICLE

Two New Indolyl Diketopiperazines, Trypostatins C and D from *Aspergillus penicillioides* Speg.

Han Zhang^{1,2} · Hong-Tao Zhu¹ · Dong Wang¹ · Chong-Ren Yang¹ · Ying-Jun Zhang^{1,3}

Received: 28 January 2018 / Accepted: 1 March 2018 / Published online: 14 March 2018
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Abstract

Twelve indolyl diketopiperazines **1–12** were isolated from the mycelia culture of *Aspergillus penicillioides* Speg., a dominant microorganism from the post fermentation process of ripe Pu-er tea. Their structures were elucidated by extensive spectroscopic methods. Among them, trypostatins C (**1**) and D (**2**) featuring with a rare methyl vinyl ketone side chain at C-2 are new compounds, while **3** and **4** were obtained for the first time from nature source. The isolates **3–12** did not show obvious cytotoxicities against five human cancer cell lines at a concentration of 40 μ M.

Keywords *Aspergillus penicillioides* Speg. · Indolyl diketopiperazines · Trypostatins

1 Introduction

Diketopiperazines (DKPs) with a scaffold of 2,5-diketopiperazine formed via the condensation of two amino acid residues. Their conformationally constrained six-membered rings made DKPs an increasing attractive pharmacophore in medicinal chemistry, with a broad pharmacological activity spectrum, e.g., antibacterial [1, 2], antifungal [3, 4], antiviral [5], anticancer [6, 7], immunosuppressive [8], neuro-protection [9], and anti-hyperglycemic [10] activities. To date, DKPs have been commonly found in the marine-derived microorganisms and endophytic fungi. The natural ones with anticancer activities were mainly isolated from *Aspergillus* and *Penicillium* genera [11]. Among which, (–)-phenylahistin

(halimide) obtained from *A. ustus* NSC-F038, was a representative DKP with a prenylated chain. Its synthetic analogue, NPI-2358, had been approved for the phase-II clinic trial for the treatment of lymphoma or solid lung cancer as a vascular disrupting agent [12].

Aspergillus penicillioides Speg. was isolated previously from the post-fermentation process of ripe Pu-er tea by our group [13]. In the research of the characteristic components responsible for health improvement of ripe Pu-er tea, the strain KIBA0502 of *A. penicillioides* was studied chemically for its metabolites. This led to the identification of 12 indolyl diketopiperazines. Trypostatins C (**1**) and D (**2**) featuring with a rare methyl vinyl ketone side chain at C-2 are new compounds, while **3** and **4** were obtained for the first time from nature source. Their structures were determined by extensive spectroscopic analysis and by comparison with literature values. Most of the isolates were tested for their cytotoxicities against five human cancer cell lines.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13659-018-0156-z>) contains supplementary material, which is available to authorized users.

✉ Ying-Jun Zhang
zhangyj@mail.kib.ac.cn

¹ State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China

² University of Chinese Academy of Sciences, Beijing 100049, China

³ Yunnan Key Laboratory of Natural Medicinal Chemistry, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China

2 Results and Discussion

The ethyl acetate extract of the mycelia culture of *A. penicillioides* (strain KIBA0502) isolated previously from the fermentative stacks of Pu-er tea was applied to repeated column chromatography over silica gel and Sephadex LH-20, followed with semi-preparative HPLC, to afford 12

Fig. 1 Structures of compounds 1–12 from *Aspergillus penicillioides*

indolyl diketopiperazines (1–12) (Fig. 1). Two of them, 1 and 2, are new compounds.

Tryprostatin C (1) was obtained as a yellow amorphous powder. Its molecular formula was determined to be $\text{C}_{21}\text{H}_{23}\text{N}_3\text{O}_4$ by the HRESIMS (m/z 404.1587 $[\text{M}+\text{Na}]^+$). The ^{13}C NMR and DEPT spectra of 1 displayed the occurrence of 21 carbon signals assignable to one ketone (δ_{C} 199.6), two amide carbonyl (δ_{C} 169.4, 165.3), one methoxy (δ_{C} 55.7), one methyl (δ_{C} 32.0), four methylenes (δ_{C} 45.6, 28.5, 25.9, 22.7), two nitrogen-bearing (δ_{C} 55.6, 59.3) and two olefinic (δ_{C} 129.5, 119.3) methines, and

eight aromatic carbons arising from a tri-substituted indolyl unit. The ^1H NMR spectrum of 1 showed the presence of one *cis*-coupled double bond (δ_{H} 6.76, 6.09, each 1H, d, $J = 12.0$ Hz, H-18, H-19), a set of ABX coupled aromatic protons [δ_{H} 7.44 (d, $J = 8.9$ Hz, H-4), 6.80 (dd, $J = 2.2$, 8.9 Hz, H-5), 6.89 (d, $J = 2.2$ Hz, H-7)] attributable to a C-6 substituted indolyl unit, two down-field shifted methines [δ_{H} 4.23 (dd, $J = 11.6$, 4.2 Hz, H-9), 3.99 (dd, $J = 7.4$, 7.5 Hz, H-12)] assignable to a 2,5-diketopiperazine skeleton, one methoxy (δ_{H} 3.81, s) and one methyl (δ_{H} 2.33, s). From the well-defined coupling sequences and spatial relationships, three sub-structural fragments could be constructed for 1, consisting of one 2,3,6-trisubstituted indole, one 2,5-diketopiperazine and one methyl vinyl ketone moieties. These were subsequently reinforced by correlative interpretation of the 2D NMR spectra (Fig. 2), which allowed the unambiguous assignment of all the ^1H and ^{13}C NMR signals. The ^1H - ^1H COSY correlations constructed obviously three partial structures of $-\text{C}_{(4)}\text{H}-\text{C}_{(5)}\text{H}-$, $-\text{C}_{(8)}\text{H}-\text{C}_{(9)}\text{H}-\text{NH}-$ and $-\text{C}_{(12)}\text{H}-\text{C}_{(13)}\text{H}_2-\text{C}_{(14)}\text{H}_2-\text{C}_{(15)}\text{H}_2-$ in 1. In the HMBC spectrum, correlation of the methoxy protons at δ_{H} 3.81 with C-6 (δ_{C} 159.7) indicated its location on C-6. The HMBC correlations of H-8 (δ_{H} 3.01, 3.78) with C-2 (δ_{C} 131.7), C-3 (δ_{C} 117.7), C-3a (δ_{C} 122.0), C-9 (δ_{C} 55.6), C-17 (δ_{C} 165.3) revealed the indole and 2,5-diketopiperazine units were linked with each other through a methylene bridge of C-8. Correlations of δ_{H} 2.33 (H_3 -21) with C-20 (δ_{C} 199.6) and C-19 (δ_{C} 119.3), and both δ_{H} 6.76 (H-18) and 6.09 (H-19) with C-20 (δ_{C} 199.6) confirmed the presence of methyl vinyl ketone partial structure, which were determined to be connected to the indolyl C-2 by the HMBC correlations of H-19 (δ_{H} 6.09) with C-2 (δ_{C} 131.7) and H-18 (δ_{H} 6.76) with C-3 (δ_{C} 117.7). Comparison of the NMR data of 1 with those of tryprostatin A [14] manifested that 1 had a similar indolyl diketopiperazine skeleton, except for the presence of a methyl vinyl ketone side chain in 1, instead of an isoprenyl group in tryprostatin A. The relative configuration of 1 was established by ROESY experiment. In which, correlation of H-9 with H-12 indicated both protons located at the same

Fig. 2 Key ^1H - ^1H COSY (black bold line) and HMBC (red arrow line from H to C) correlations of compound 1

side. Based on comparison of its specific rotation with that of tryprostatin A [15], the absolute stereochemistry at C-9 and C-12 in **1** was assigned to be both as *S* configurations, which are the same to those of the other known 2,5-diketopiperazine-type compounds. On the basis of the above evidences, the structure of tryprostatin C (**1**) was deduced as shown in Fig. 1.

Compound **2** obtained as yellow amorphous powder, had a molecular formula of $C_{21}H_{23}N_3O_4$, as determined by the HRESIMS (m/z 404.1581 $[M+Na]^+$), which was same to that of **1**. The 1D NMR spectroscopic data were almost the same with those of **1**, suggesting that **2** had the same indole diketopiperazine skeleton. The only different between **2** and **1** was the appearance of a *trans*-coupled double bond [$(\delta_H$ 7.48, 6.36, each 1H, $dJ = 16.0$ Hz, H-18, H-19)] in **2**, instead of the *cis* form in **1**. The 2D NMR spectra further revealed that structure of **2** as shown in Fig. 1. The configuration of **2** was deduced to be the same as that of **1**, according to the ROESY correlation of H-9 with H-12, and further confirmed by the specific rotation. Therefore, the absolute configuration of tryprostatin D (**2**) was established to be 9*S*,12*S*.

It is noted, at the room temperature, compound **2** changed rapidly to **1** in a methanol or chloroform solutions. The *cis* conformation of double bond in **1** is obviously more stable than the *trans* form in **2**. This is opposite to the most cases that *trans*-coupled double bonds are more stable than the *cis* ones. It might be the formation of an intramolecular hydrogen bond between 1-NH and the C-20 ketone that caused the C-18/C-19 double bond to be *cis* form in **1**.

Compounds **3–12** were 10 known indolyl diketopiperazines. They were identified as methyl(*S*)-2-((3*R*,4*aS*,9*aR*)-hydroxy-2-oxo-3-(4-oxoquinazolin-3(4*H*)-yl)-2,3,4,4*a*,9,9*a*-hexahydro-1*H*-pyrido[2,3-*b*]indol-1-yl)propanoate (**3**), methyl(*S*)-2-((3*R*,4*aR*,9*aS*)-4*a*-hydroxy-2-oxo-3-(4-oxoquinazolin-3(4*H*)-yl)-2,3,4,4*a*,9,9*a*-hexahydro-1*H*-pyrido[2,3-*b*]indol-1-yl)propanoate (**4**) [16, 17], chaetominine (**5**) [18, 19], brevianamide F (**6**) [20], 6-methoxyspirotryprostatin B (**7**) [21], verruculogen (**8**) [22], fumitremorgin C (**9**) [23], cyclotryprostatin A (**10**), cyclotryprostatin B (**11**) and cyclotryprostatin C (**12**) [24], respectively, by comparison of their spectroscopic data with literature values, and single-crystal X-ray diffraction in the case of **5**. All of the isolates were obtained from *A. penicilliodes* for the first time.

The known compounds **3–12** were evaluated for their cytotoxic activities against human myeloid leukemia HL-60, hepatocellular carcinoma SMMC7721, lung cancer A-549, breast cancer MCF-7, and colon cancer SW480 cell lines. However, no compounds showed obvious activity at a concentration of 40 μ M.

3 Experimental

3.1 General Experimental Procedures

Optical rotations were measured with a P-1020 polarimeter (JASCO, Tokyo, Japan). UV spectra were recorded on a Shimadzu UV2401A ultraviolet–visible spectrophotometer. HREIMS spectra were run on a Waters Autospec Premier P776. NMR spectra were measured in CD_3OD and recorded on a Bruker DRX-600 spectrometer, using TMS as an internal standard. Chemical shifts were reported in units of δ (ppm) and coupling constants (J) were expressed in Hz. Column chromatography (CC) were carried out silica gel (200–300 mesh, Qingdao Haiyang Chemical Co. Ltd., Qingdao, China), Sephadex LH-20 (25–100 μ m, GE Healthcare Bio-Science AB). An Agilent series 1260 (Agilent Technologies) were used for semi-preparative HPLC with an Agilent ZORBAX SB-C18 column (5 μ m, 250 mm $\times$ 9.4 mm). TLC was performed on pre-coated TLC plates (0.2–0.25 mm thickness, GF254 Silica gel, Qingdao Hailang Chemical Co. Ltd., Qingdao, China) with compounds visualized by spraying with anisaldehyde-sulfuric acid reagent and careful heating.

3.2 Fungal Material

Aspergillus penicilliodes Speg. was isolated previously by our group from the fermentative stacks of Pu-er tea produced at Puer specific tea factory in Puer city, Yunnan Province, China, and deposited in China general microbiological culture collection center (CGMCC), Institute of Microbiology, Chinese Academy of Sciences [13]. A loop of spores from a colony growing on potato dextrose agars was inoculated into potato dextrose seed broth (20 g glucose, 200 g potato, 4 g peptone, 0.5 g $MgSO_4$, 1.0 g KH_2PO_4 , and 1000 mL distilled water) for 3 days in the dark at 28 $^{\circ}C$, 140 r/min. The seed culture (10 mL) was inoculated into 100 $\times$ 500 mL Erlenmeyer flasks, each containing 100 g of wheat immersed in water and sterilized. Fermentation was conducted under stationary condition for 20 days in the dark at 28 $^{\circ}C$.

3.3 Extraction and Isolation

The mycelia culture was extracted three times with $CHCl_3$ –MeOH (1:1 v/v). After concentrated in vacuo, the resultant extract was suspended into H_2O and partitioned successively with petroleum ether (4 $\times$ 3 L), EtOAc (4 $\times$ 3 L), and *n*-BuOH (4 $\times$ 3 L). The EtOAc fraction (40.5 g) was subjected to a silica gel column, eluting with $CHCl_3$ –MeOH (100:0, 98:2, 95:5, 90:10, 85:15, 80:20 v/v) to afford six fractions (Fr. 1–6), which were combined based

Table 1 ^1H and ^{13}C NMR spectroscopic data (in CDCl_3) of **1** and **2**. (δ in ppm)

No.	1		2	
	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
1		8.33 (s)		8.28 (s)
2	131.7		131.7	
3	117.7		117.8	
3a	122.0		122.0	
4	120.2	7.44 d (8.9)	120.2	7.44 d (8.9)
5	112.9	6.80 dd (2.2, 8.9)	112.9	6.80 dd (2.2, 8.9)
6	159.7		159.8	
7	94.1	6.89 d (2.2)	94.1	6.89 d (2.2)
7a	137.7		137.7	
8	25.9	3.01 dd (11.2, 15.1) 3.78 dd (4.0, 14.8)	26.0	3.01 dd (11.0, 15.1) 3.78 dd (4.1, 15.2)
9	55.6	4.23 dd (11.6, 4.2)	55.7	4.23 dd (11.6, 4.2)
10		5.41 s		
11	169.4		169.5	
12	59.3	3.99 dd (7.4, 7.5)	59.4	3.99 dd (7.4, 7.5)
13	28.5	1.96 m, 2.27 m	28.5	1.96 m, 2.27 m
14	22.7	1.97 m, 1.84 m	22.8	1.97 m, 1.84 m
15	45.6	3.54 m, 3.59 m	45.7	3.54 m, 3.59 m
16				
17	165.3		165.3	
18	129.5	6.76 d (12.0)	129.6	7.48 d (16.0)
19	119.3	6.09 d (12.0)	119.3	6.36 d (16.0)
20	199.6		199.3	
21	32.0	2.33 s	32.1	2.33 s
OCH_3	55.7	3.81 s	55.7	3.81 s

on TLC analyses. Further separation of Fr.3 (11.0 g) by silica gel CC, using CHCl_3 –MeOH (100, 100:1, 100:2 v/v) as the eluent afforded 12 sub-fractions (subfr.3-1-subfr.3-12). Subfr.3-3 (30 mg) and subfr.3-5 (0.5 g) were separately purified by semi-preparative HPLC (70% MeOH– H_2O) to give **1** (2.7 mg) and **2** (1.3 mg), and **3** (3.2 mg), **4** (5.0 mg) and **5** (80 mg), respectively. Fr.4 (8.0 g) was applied to Sephadex LH-20 CC, eluting with MeOH– CHCl_3 (1:1) to afford five sub-fractions (subfr.4-1-subfr.4-5). Subfr.4-3 and Subfr.4-6 were separately subjected to silica gel CC, eluting with a gradient of increasing acetone (0–100%) in petroleum ether to afford subfr. 4-3-1-subfr.4-3-7 and subfr.4-6-1-subfr.4-6-8, respectively. Semi-preparative HPLC (60% aq. MeOH) purification of subfr.4-3-2 (60 mg) and subfr. 4-6-5 (150 mg) afforded **6** (7.0 mg), and **9** (8.2 mg), **10** (23 mg) and **11** (3.0 mg), respectively. Fr.5 (5.5 g) was separated into eight sub-fractions (subfr.5-1-subfr.5-8) by Sephadex LH-20 CC (MeOH– CHCl_3 , 1:1). Subfr.5-4 (62 mg) was purified by semi-preparative HPLC (50% aq. MeOH) to yield **7** (6.4 mg), **8** (2.0 mg) and **12** (5.0 mg).

3.4 Spectroscopic Data

3.4.1 Trypostatin C (**1**)

Yellow amorphous powder; $[\alpha]_{\text{D}}^{20}$ –24.5 (c 0.27, CHCl_3); UV (MeOH) λ_{max} (log ϵ): 202 (4.44), 219 (4.13), 398 (3.86) nm; ^1H and ^{13}C NMR data see Table 1; ESI–MS (positive ion mode): m/z 404 $[\text{M}+\text{Na}]^+$; HRESIMS (positive ion mode): m/z 404.1586 $[\text{M}+\text{Na}]^+$, (calcd. for $\text{C}_{20}\text{H}_{37}\text{NO}_6\text{Na}$, 404.1586).

3.4.2 Trypostatin D (**2**)

Yellow amorphous powder; $[\alpha]_{\text{D}}^{20}$ –61.7 (c 0.13, CHCl_3); UV (MeOH) λ_{max} (log ϵ): 201 (4.37), 217 (4.18), 400 (3.94) nm; ^1H and ^{13}C NMR data see Table 1; ESI–MS (positive ion mode): m/z 404 $[\text{M}+\text{Na}]^+$; HRESIMS (positive ion mode): m/z 404.1581 $[\text{M}+\text{Na}]^+$, (calcd. for $\text{C}_{22}\text{H}_{39}\text{NO}_6\text{Na}$, 404.1586).

3.5 Cytotoxicity Assay

Five human cancer cell lines, myeloid leukemia HL-60, hepatocellular carcinoma SMMC7721, lung cancer A-549 cells, breast cancer MCF-7, and colon cancer SW480, were used in the cytotoxic assay. All the cells were cultured in RPMI-1640 or DMEM medium (Hyclone, USA), supplemented with 10% fetal bovine serum (Hyclone, USA). The cytotoxic assay was performed according to the MTS method in 96-well microplates. Briefly, adherent cells (100 μ L) was seeded into each well of 96-well cell culture plates and allowed to adhere for 24 h before drug addition, while suspended cells were seeded just before drug addition, each tumor cell line was exposed to the test compound dissolved in DMSO in triplicates for 48 h at 37 °C, with DDP and taxol (Sigma, USA) as positive controls. After the incubation, 20 μ L MTS and 100 μ L medium was added to each well after removal of 100 μ L medium, and the incubation continued for 4 h at 37 °C. The optical density was measured at 492 nm using a Multiskan FC plate reader (Thermo Scientific, USA).

4 Conclusion

In this study, 12 indole diketopiperazines **1–12** were identified for the first time from *A. penicilliodes*. Among them, **1** and **2** are new compounds, while **3** and **4** are new natural products. So far, the natural diketopiperazines with anti-cancer activities were mainly isolated from *Aspergillus* and *Penicillium* genera, with prenylated side chain as the most representative structural fragment, accounting for the majority of this class of compounds. This is the first time to have obtained indole diketo-piperazines featuring with a methyl vinyl ketone side chain at C-2.

Acknowledgements This work was supported by the National Natural Science Foundation of China (No. 21672223). The authors are grateful to the staffs of the analytical and bioactivity screening groups at State Key Laboratory of Phytochemistry and Plant Resources in West China, KIB, CAS, for measuring the spectroscopic data and bioactivities.

Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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