








New Cyclic Peptides from the Endophytic *Aspergillus versicolor* 0312 with Their Antimicrobial Activity

Yanping Li ¹Ψ, Shanling Sheng ²Ψ, Jian Feng ², Yudan Wang ²,
Jing Guo ¹, Yuntao Jiang ^{2*} and Weiguang Wang ^{2*}

¹ School of Chinese Pharmacy, Yunnan University of Chinese Medicine, Kunming 650500, P. R. China

² Key Laboratory of Chemistry in Ethnic Medicinal Resources, State Ethnic Affairs Commission and Ministry of Education, Yunnan Minzu University, Kunming 650031, P. R. China

(Received December 17, 2021; Revised March 28, 2022; Accepted March 29, 2022)

Abstract: This article aims to investigate the chemical constituents of the endophytic *Aspergillus versicolor* 0312 cultivated in the solid fermentation of rice perlite. Two new cyclic peptide compounds, 7-hydroxydehydrocyclopeptin (**1**), 14,31-dimethoxy-penicopeptide A (**2**) and seven known compounds were isolated from the fermentation. Their structures were characterized by using 1D and 2D NMR techniques and MS spectrometry methods. The antibacterial effects of the isolated compounds were evaluated and the results showed compound **2** exhibited moderate antimicrobial activities against *Bacillus subtilis*.

Keywords: *Aspergillus versicolor* 0312; endophytic fungi; cyclic peptide; antimicrobial activity. © 2022 ACG Publications. All rights reserved.

1. Introduction

Endophytes are bacterial or fungal microorganism lived inside the healthy tissues of the host plant, typically causing no apparent symptoms of disease [1]. As a poorly investigated store of microorganisms ‘hidden’ within the host plants, endophytes are obviously a rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential. The number of secondary metabolites produced by fungal endophytes is larger than that of any other endophytic microorganism class [2]. Natural products from fungal endophytes have a broad spectrum of biological activity, and they can be grouped into several categories, including alkaloids, steroids, terpenoids, isocoumarins, quinones, phenylpropanoids and lignans, phenol and phenolic acids, aliphatic metabolites, lactones, etc [3]. These bioactive secondary metabolites have the great potential to become new drug source molecules [4, 5]. *Paris polyhylla* var. *yunnanensis* (Franch) Hand-Mazz, a characteristic medicinal plant in Yunnan Province, has been used for medicinal purpose for a long time [6]. It is the core raw material of many kinds of proprietary Chinese medicines, such as

ΨYanping Li and Shanling Sheng contributed equally.

*Corresponding authors: E-Mails: ymujyt_2018@163.com; Phone: 086-871-65926940 (Y. Jiang); wwg@live.cn; Phone: 086-871-65920586 Fax: 086-871-65920216 (W. Wang).

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"Gongxuening capsule", and "Yunnan Baiyao aerosol" [7]. At present, the development of *P. polyohylla* is facing many problems, such as the rapid consumption of wild resources and the difficulty of artificial seedling breeding technology [8]. All these make the research on endophytes of *P. polyohylla* great significance for its resource protection and development and utilization.

In order to explore the secondary metabolites with biological activities from endophytic fungi in *P. polyohylla*, the solid fermentation of endophytic *Aspergillus versicolor* 0312 were studied and nine compounds, including two new ones were obtained (Figure 1).

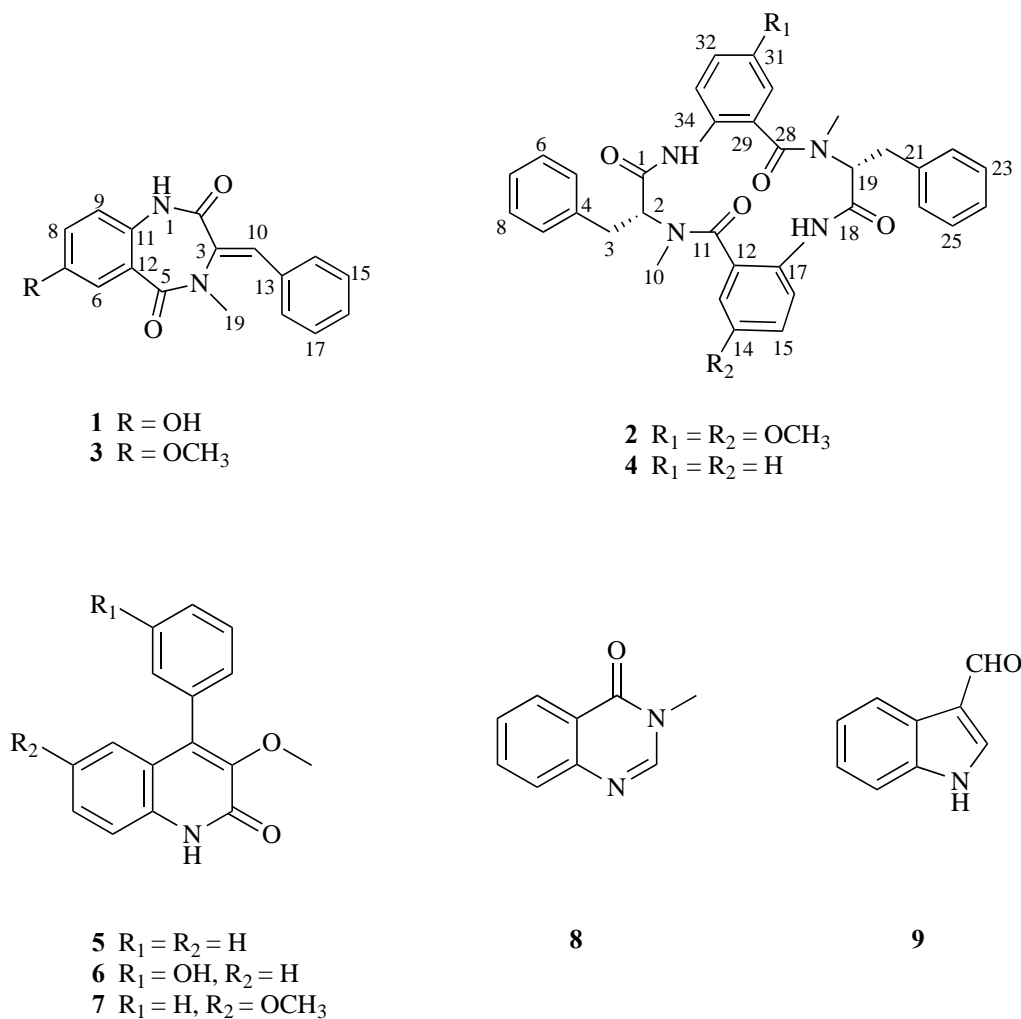


Figure 1. Structures of compounds 1-9

2. Materials and Methods

2.1. General Experimental Procedures

Optical rotations were measured in MeOH on a JASCO P-1020 polarimeter. ¹H NMR (800 MHz) and ¹³C NMR (200 MHz) spectra were measured at 25 °C on a Bruker AM 800 NMR spectrometer. HR-ESI-MS were recorded on a Bruker-HCT/Esquire 3000. TLC was performed using Qingdao Marine Chemistry Ltd precoated plates (Silica gel GF254, Qingdao Marine Chemistry Ltd., China). Prep. HPLC was performed on an Agilent-1260 system equipped with a ZORBAX SB-C₁₈ column (4.6 mm × 250 mm, Agilent Technologies, USA).

2.2. Endophyte Strain

The endophyte strain isolated from the rhizome of *P. polyohylla* was identified as *Aspergillus versicolor* 0312 [9, 10]. A voucher specimen was stored in the State Ethnic Affairs Commission & Ministry of Education, Yunnan University of Nationalities.

2.3. Fermentation and Isolation

The endophytic *Aspergillus versicolor* 0312 cultivated in the solid fermentation of rice perlite was partitioned successively with equal volume of ethyl acetate for three times. After removal of the solvent under vacuum, the ethyl acetate portion (216 g) was fractionated by a silica gel column, eluted with CH₂Cl₂/EtOAc (100:0→0:100) to give seven eluents (*Fr.* 1~7). Then fraction *Fr.*5 (12.5 g) was subjected to silica gel column chromatography with CH₂Cl₂/MeOH (100:1→1:1) and five subfractions were obtained *Fr.*5-1~*Fr.*5-5. Compounds **2** (3.5 mg), **4** (4.5 mg), **5** (4.0 mg) **6** (4.9 mg) and **7** (3.0 mg) were obtained by preparative HPLC (ACN-H₂O 50:50, flow rate 3 mL/min) from *Fr.*5-1. Subfraction (*Fr.*5-2) was purified by preparative HPLC (ACN-H₂O 70:30, flow rate 3 mL/min) to get compound **1** (3.4 mg), **3** (3.9 mg) and **9** (4.0 mg). The subfraction *Fr.*6 (11 g) was further separated by silica gel column chromatography with CH₂Cl₂/MeOH (100:1→1:1) to give six subfractions (*Fr.*6-1~*Fr.*6-6). The subfraction *Fr.*6-3 was further separated by C18 column with MeOH-H₂O (30:50→95:5) to obtain subfractions *Fr.* 6-3-1~6. The subfraction *Fr.* 6-3-5 was further purified by preparative HPLC with ACN-H₂O 45:55, flow rate 3 mL/min to yield **8** (2.5 mg).

2.4. Antimicrobial Bioassays

Antibacterial and antifungal assays were conducted in triplicate followed the National Center for Clinical Laboratory Standards recommendations. The bacteria strains, *Staphylococcus aureus* (CGMCC 1.2465), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 6633) and *Escherichia coli* (CGMCC 1.3373) were grown on Mueller-Hinton agar (MHA). Targeted microbes (3-4 colonies) were prepared from broth culture (bacteria: 37 °C for 24 h), and the final spore suspensions of bacteria (in MHB medium) was 10⁶ cells/mL. Test compounds at different concentrations were transferred into 96-well clear plate in triplicate, and the suspensions of the test strains were added to each well respectively, achieving a final volume of 200 μL. For antibacterial tests, the absorbance at 595 nm was measured using a microplate reader (TECAN) after incubation (37 °C for 24 h), and ampicillin was used as the positive control. The inhibition rate was calculated and plotted versus test concentrations to afford the IC₅₀ value. The MIC was defined as the lowest test concentration that completely inhibited the growth of the test organisms.

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was obtained as slight yellow oil. The HR-ESI-MS of **1** showed a quasimolecular ion [M + H]⁺ at *m/z* 295.1077 (calcd. for [C₁₇H₁₅N₂O₃]⁺ 295.1077), consistent with a molecular formula C₁₇H₁₄N₂O₃, indicating 12 degrees of unsaturation. The ¹H NMR spectrum showed signals for a trisubstituted benzene unit at δ_H 6.96 (1H, d, *J* = 8.6 Hz, H-9), δ_H 7.25 (1H, d, *J* = 1.3 Hz, H-6) and δ_H 6.96 (1H, dd, *J* = 8.6, 2.1 Hz, H-8) as well as one olefinic proton at δ_H 6.87 (1H, s, H-10). What's more, a methyl signal appeared at δ_H 3.10 (3H, s, H-19) indicated the connection with nitrogen atom. The ¹³C NMR and DEPT spectra of compound **1** displayed 17 carbon signals including twelve carbons signals on benzene ring (Table 1). The remaining five carbon signals belongs to two olefinic carbons at δ_C 135.7 (C-3), 131.3 (C-10), two amide groups at δ_C 172.3 (C-2), 169.0 (C-5), and one methyl group at δ_C 36.1 (C-19). Analyses of the NMR data suggested that compound **1** shared a

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structure similar to that of 7-methoxydehydrocyclopeptin (compound **3**) [11]. The only difference between the two compounds was the substitution of C-7. The hydroxyl substitution at C-7 of compound **1** was confirmed by high resolution mass spectrum data and HMBC spectrum, which showed the correlations between the aromatic protons H-8 [δ_{H} 6.96 (1H, dd, $J = 8.6, 2.1$ Hz)], H-9 [δ_{H} 6.96 (1H, d, $J = 8.6$ Hz)], H-6 [δ_{H} 7.25 (1H, d, $J = 1.3$ Hz)] and C-7 (δ_{C} 156.3) (Figure 2). The NOE correlations between H-19 (δ_{H} 3.10) and H-18 (δ_{H} 7.34) revealed a trans double bond between C-10 and C-3 in compound **1**. Finally, the structure of compound **1** was elucidated as 7-hydroxydehydrocyclopeptin.

Compound **2** was obtained as a white powder. The molecular formula $\text{C}_{36}\text{H}_{36}\text{N}_4\text{O}_6$ was established by the HR-ESI-MS at m/z 621.2704 [$\text{M}+\text{H}$] $^+$ (calcd. for $[\text{C}_{36}\text{H}_{37}\text{N}_4\text{O}_6]^+$ 621.2701). The ^1H NMR displayed two methoxy signals at δ_{H} 3.88 (3H, s, 14-OCH $_3$) and δ_{H} 3.83 (3H, s, 31-OCH $_3$), two amide N-methyl signals at δ_{H} 2.92 (3H, s, H-10) and δ_{H} 3.07 (3H, s, H-27), and two amino acid protons at δ_{H} 4.29 (1H, dd, $J = 10.5, 7.1$ Hz, H-2) and δ_{H} 4.42 (1H, t, $J = 7.6$ Hz, H-19). The ^{13}C NMR and DEPT spectra indicated that compound **2** possessed 36 carbon signals, involving two methoxyls, two N-methyls, two methylenes, eighteen methines and twelve quaternary carbons (Table 2). Analyses of the NMR data indicated compound **2** was a cyclic tetrapeptide skeleton compound containing two PHE (phenylalanine) groups and two aminobenzoic acid moieties, which was confirmed by HMBC correlations from H-10 [δ_{H} 2.92 (3H, s)] to C-2 (δ_{C} 69.9) and C-11 (δ_{C} 168.0), H-2 [δ_{H} 4.29 (1H, dd, $J = 10.5, 7.1$ Hz)] to C-11 (δ_{C} 168.0), H-27 [δ_{H} 3.07 (3H, s)] to C-19 (δ_{C} 57.9) and C-28 (δ_{C} 170.4), and H-19 [δ_{H} 4.42 (1H, t)] to C-28. Compound **2** was elucidated as a tetrapeptide by comparison of the ^1H and ^{13}C NMR data with those of penicopeptide A (**4**) [12]. Compared with penicopeptide A, two methoxy groups at C-14 and C-31 in compound **2** were also confirmed in HSQC by the correlations from 14-OCH $_3$ (δ_{H} 3.88) to C-14 and 31-OCH $_3$ (δ_{H} 3.83) to C-31 and HMBC correlations from both methoxy hydrogen signals to C-14 and C-31, respectively. Thus, compound **2** was identified as 14, 31-dimethoxy-penicopeptide A. (Figure 2) Usually, the symmetrical compound should show only one group of NMR signals. While penicopeptide A was proved to exhibit two different unit of ^1H and ^{13}C NMR data for the asymmetrical conformations even though it is a symmetrical tetrapeptide [12]. Therefore, compound **2** might be also the similar situation of penicopeptide A.

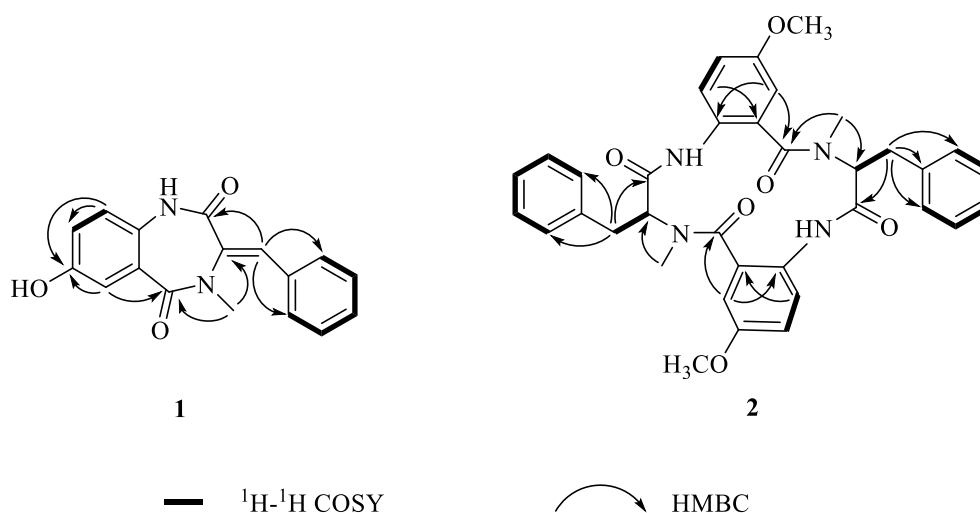
The structures of known compounds were characterized as 7-methoxydehydrocyclopeptin (**3**) [11], penicopeptide A (**4**) [12], 3-*O*-methylviridicatin (**5**) [13], 3-*O*-methylviridicatol (**6**) [13], 3,6-*O*-dimethylviridicatin (**7**) [13], 3-methylquinazolin-4 (3*H*)-one (**8**) [14] and 1*H*-indole-3-carboxaldehyde (**9**) [15], by comparison of their ^1H and ^{13}C NMR data with those reported data.

Table 1. ^1H NMR and ^{13}C NMR data of compound **1** (800 MHz and 200 MHz, CD_3OD)

No.	δ_{C}	δ_{H} (mult, J , Hz)	No.	δ_{C}	δ_{H} (mult, J , Hz)
2	172.3 (C)		12	127.9 (C)	
3	135.7 (C)		13	133.7 (C)	
5	169.0 (C)		14	130.2 (CH)	7.34 (1H,m)
6	116.9 (CH)	7.25 (1H, d, 1.3)	15	130.2 (CH)	7.41 (1H,m)
7	156.3 (C)		16	130.9 (CH)	7.38 (1H, t, 7.3)
8	121.7 (CH)	6.96 (1H, d, 8.6)	17	130.2 (CH)	7.41 (1H, m)
9	123.7 (CH)	6.96 (1H, d, 8.6)	18	130.2 (CH)	7.34 (1H, m)
10	131.3 (CH)	6.87 (1H, s)	19	36.1 (CH $_3$)	3.10 (3H, s)
11	129.7 (C)				

Table 2. ^1H NMR and ^{13}C NMR data of compound **2** (800 MHz and 200 MHz, CD_3OD).

No.	δ_{C}	δ_{H} (mult, J, Hz)	No.	δ_{C}	δ_{H} (mult, J, Hz)
1	172.0 (C)		18	171.0 (C)	
2	69.9 (CH)	4.29 (1H, dd, 10.5, 7.1)	19	57.9 (CH)	4.42 (1H, t, 7.6)
3	35.1 (CH_2)	2.69 (1H _a , dd, 13.4, 10.7) 2.79 (1H _b , dd, 13.4, 7.0)	20	32.9 (CH_2)	3.25 (1H _a , dd, 14.5, 7.2) 3.39 (1H _b , dd, 14.5, 7.9)
4	137.3 (C)		21	138.2 (C)	
5	130.0 (CH)	7.04 (1H, d, 7.3)	22	130.0 (CH)	7.23 (1H, m)
6	129.8 (CH)	7.23 (1H, m)	23	129.6 (CH)	7.27 (1H, m)
7	128.2 (CH)	7.23 (1H, m)	24	127.8 (CH)	7.17 (1H, m)
8	129.8 (CH)	7.23 (1H, m)	25	129.6 (CH)	7.27 (1H, m)
9	130.0 (CH)	7.04 (1H, d, 7.3)	26	130.0 (CH)	7.23 (1H, m)
10	39.8 (CH_3)	2.92 (3H, s)	27	29.6 (CH_3)	3.07(3H, s)
11	168.0 (C)		28	170.4 (C)	
12	128.8 (C)		29	129.2 (C)	
13	115.3 (CH)	7.44 (1H, d, 2.8)	30	114.6 (CH)	7.31 (1H, d, 2.8)
14	158.1 (C)		31	158.1 (C)	
14-O CH_3	56.2 (CH_3)	3.88 (3H, s)	31- O CH_3	56.1 (CH_3)	3.83 (3H, s)
15	120.9 (CH)	7.19 (1H, m)	32	121.0 (CH)	7.11 (1H, m)
16	123.3 (CH)	7.10 (1H, m)	33	123.7 (CH)	7.01 (1H, d, 8.8)
17	137.2 (C)		34	138.2 (C)	

**Figure 2.** ^1H - ^1H COSY and selected HMBC of compounds **1** and **2**

3.2. Antimicrobial Activity

The antibacterial effects of compounds **1-9** were evaluated by following the National Center for Clinical Laboratory Standards (NCCLS) recommendations [16]. Compound **2** showed potent antibacterial activity against *Bacillus subtilis* bacteria *in vitro* with IC_{50} values at $31.89 \mu\text{M}$ compared with that of positive control ampicillin ($27.38 \mu\text{M}$). Other compounds are generally weakly active or completely inactive against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Escherichia coli* with IC_{50} values greater than $70 \mu\text{M}$.

Acknowledgments

This project was supported financially by the National Natural Science Foundation of China and Yunnan (Nos. 31960095 and 202101AS070022); Yunnan Ten-thousand Talents Program to W.-G.W and Y.-P.L.; the Union Special Fund of Yunnan Provincial Science and Technology Department and Yunnan University of Chinese Medicine [No. 2019FF002(-005)].

Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

ORCID

Yanping Li : [0000-0002-2409-3569](https://orcid.org/0000-0002-2409-3569)

Shanling Sheng: [0000-0002-4615-3315](https://orcid.org/0000-0002-4615-3315)

Jian Feng: [0000-0001-7849-7481](https://orcid.org/0000-0001-7849-7481)

Yudan Wang: [0000-0001-8072-7944](https://orcid.org/0000-0001-8072-7944)

Jing Guo: [0000-0001-8315-6147](https://orcid.org/0000-0001-8315-6147)

Yuntao Jiang: [0000-0001-6320-6506](https://orcid.org/0000-0001-6320-6506)

Weiguang Wang: [0000-0003-3395-767X](https://orcid.org/0000-0003-3395-767X)

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