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ORIGINAL ARTICLE

Trichopeptides A and B, trichocyclodipeptides A–C, new peptides from the ascomycete fungus *Stagonospora trichophoricola*

Zhengkun Chen[1,2], Xiuli Xu[1], Jinwei Ren[2], Wenzhao Wang[2], Xingzhong Liu[2] and Erwei Li[2]

Trichopeptides A (1) and B (2), new linear tetrapeptide and tripeptide, respectively, and three new diketopiperazines trichocyclodipeptides A–C (3–5) were isolated from the fermentation of the ascomycete fungus *Stagonospora trichophoricola*, a fungus isolated from the soil sample surrounding the fruiting body of *Ophiocordyceps sinensis* in Maqin Country, Qinghai Province, People's Republic of China. Their structures were primarily elucidated by interpretation of NMR and MS experiments. The absolute configurations of 1–5 were assigned through Marfey's method on their acid hydrolyzates. Compound 3 showed antifungal activity against *Candida albicans* with the IC_{50} and MIC values of 22 and 90 μg ml^{-1}, respectively.
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INTRODUCTION

Fungi growing in special environments tends to produce bioactive secondary metabolites with plenty of structure characteristics because of their evolutional and differential metabolic systems that highly adapted during the process of natural selection.[1–4] In recent years, many bioactive compounds were discovered from *Ophiocordyceps*-colonizing fungi and *Ophiocordyceps*-related fungi isolated from the soil sample surrounding the fruiting body of *O. sinensis*, which inhabits low temperature and high altitude environments.[5–8] In our course of investigating new bioactive natural products from the above fungi, a strain *S. trichophoricola* (P068) was isolated from the soil sample in Maqin Country, Qinghai Province, People's Republic of China, which surrounded the fruiting body of *O. sinensis*. Some noteworthy examples of bioactive compounds have been found from the *Stagonospora* genus: elsinochrome A and leptosphaerodione,[9] two phytotoxin polyketides isolated from *Stagonospora convolvuli* (LA39); stagonolide H,[10] a toxin from fungal pathogen *Stagonospora cirsii*; dihydromaldoxin,[11] an endothelin receptor antagonist from *Stagonospora* sp.; and pramanicin,[12] an antimicrobial agent from *Stagonospora* sp.

Through chemical investigation of the extract from the fermentation of *S. trichophoricola*, five new peptides, trichopeptide A (**1**), a linear tetrapeptide, trichopeptide B (**2**), a linear tripeptide and trichocyclodipeptides A–C (**3–5**), and three diketopiperazines were obtained. Details of the isolation, structure elucidation and antimicrobial activities of these compounds are reported herein.

RESULTS AND DISCUSSION

Trichopeptide A (**1**) was obtained as a brown gum. It has a molecular formula of $C_{27}H_{42}N_4O_7$ (nine degrees of unsaturation), on the basis of its HR–ESI–MS pseudomolecular ion peak ($[M+H]^+$ at *m/z* 535.3124, calcd for 535.3126). Analysis of its ^1H and ^{13}C NMR data (Table 1) revealed the presence of three amide *N*-H protons (δ_H 7.59, 7.73 and 8.45), six methyl groups including one *N*-methyl (δ_H 3.11 and δ_C 31.1), four methylenes, six methines including three *N*-methines, one monosubstituted benzene ring and five carboxylic carbons (δ_C 168.9, 170.4, 171.1, 173.0 and 175.0). These data accounted for all the nine degrees of unsaturation. Interpretation of COSY of **1** identified five proton spin systems, which were C-2-C-6, *N*H-C-*γ* (homoserine, Hse), *N*H-C-*β* (alanine, Ala), C-*α*-C-δ/C-*δ*′ (*N*-methyl leucine, *N*-MeLeu) and *N*H-C-δ/C-*γ*′ (isoleucine, Ile). Interpretation of its ^1H, ^{13}C NMR and HMBC data, the amino acid residues of Hse, Ala, *N*-MeLeu and Ile were established. The amino acid sequence of **1** was deduced to be Ile-*N*-MeLeu-Ala-Hse by analyses of the HMBC correlations of *N*H ($\delta_{H\text{-Ile}}$ 7.73) to C ($\delta_{C\text{-}N\text{-MeLeu}}$ 170.4), *N*-Me ($\delta_{H\text{-}N\text{-MeLeu}}$ 3.11) to C ($\delta_{C\text{-Ala}}$ 173.0) and *N*H ($\delta_{H\text{-Ala}}$ 8.45) to C ($\delta_{C\text{-Hse}}$ 171.1). The HMBC correlations from protons H-2/H-6 (δ_H 7.82) and the *N*-H of Hse (δ_H 7.59) to C-7 (δ_C 168.9) showed the connection of the linear peptide to the benzoyl group. Thus, the planar structure of trichopeptide A (**1**) was established (Figure 2).

Marfey's method was applied to identify the absolute configurations of the amino acid residues derived from the acid hydrolysis of **1**.

[1]School of Ocean Sciences, China University of Geosciences, Beijing, People's Republic of China and [2]State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, People's Republic of China
Correspondence: Dr X Xu, School of Ocean Sciences, China University of Geosciences, No.29 Xueyuan Road, Haidian District, Beijing 100083, People's Republic of China.
E-mail: xuxl@cugb.edu.cn
or Dr E Li, State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, No.1 West Beichen Road, Chaoyang District, Beijing 100101, People's Republic of China.
E-mail: liew@im.ac.cn
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Table 1 NMR spectral data of compounds 1 and 2

		1 *in* $CDCl_3$			***2*** *in Acetone-*d_6		
Unit	*Position*	δ_C[a] *type (δ in p.p.m.)*	δ_H[b] *(J in Hz)*	*HMBC (H→C)*	δ_C[a]*, type (δ in p.p.m.)*	δ_H[b] *(J in Hz)*	*HMBC (H→C)*
	1	133.0, qC			135.2, qC		
	2	127.3, CH	7.82, d (7.5)	4, 6, 7	128.2, CH	7.91, d (7.5)	4, 6, 7
	3	128.9, CH	7.45, t (7.5,7.4)	1, 5, 7	129.3, CH	7.47, t (7.5,7.4)	1, 5, 7
	4	132.4, CH	7.53, d (7.1)	2, 6	132.3, CH	7.54, d (7.1)	2, 6
	5	128.9, CH	7.45, t (7.5,7.4)	1, 3, 7	129.3, CH	7.47, t (7.5,7.4)	1, 3, 7
	7	168.9, qC			167.6, qC		
Hse	CO	171.1, qC			171.5, qC		
	α	49.8, CH	5.00, m	7, *β*, *γ*, CO	52.5, CH	4.78, m	7, *β*, *γ*, CO
	β	36.9, CH_2	2.37, m, 1.59, m	*α*, CO	35.7, CH_2	2.15, m, 1.97, m	*α*, *γ*, CO
	γ	58.6, CH_2	3.83, m, 3.63, m	*α*, *β*	59.4, CH_2	3.71, m	*α*
	*N*H		7.59, d (6.8)	7			
Ala	CO	173.0, qC			173.2, qC		
	α	46.2, CH	5.02, m	*β*, CO	46.4, CH	4.92, m	*β*, CO
	β	18.8, CH_3	1.3, d (6.7)	*α*, CO	18.5, CH_3	1.29, d (6.7)	*α*, CO
	*N*H		8.45, d (6.8)	CO (Hse)			
N-MeLeu	CO	170.4, qC			173.8, qC		
	α	54.9, CH	5.38, t (7.0, 7.6)	CO (Ala), *N*-Me, *γ*, *β*, CO	55.4, CH	5.18, t (7.1,7.6)	CO (Ala), *N*-Me, *γ*, *β*, CO
	β	37.7, CH_2	1.82, m, 1.47, m	*α*, *γ*, *δ*, CO	38.1, CH_2	1.84, m, 1.73, m	*γ*, *δ*
	γ	25.4, CH	1.48, m	*β*	25.7, CH	1.49, m	
	δ	22.7, CH_3	0.83, d (6.3)	*γ*, *β*	21.5, CH_3	0.88, d (6.3)	*β*, *δ′*
	δ′	23.4, CH_3	0.87, d (6.3)	*β*, *γ*, *δ*	23.6, CH_3	0.94, d (6.3)	*β*, *γ*, *δ*
	N-Me	31.1, CH_3	3.11, s	*α*, *β*, CO (Ala)	31.6, CH_3	3.06, s	*α*, CO(Ala)
Ile	CO	175.0, qC					
	α	55.9, CH	4.68, m	*β*, *γ*, *γ′*, CO, CO (*N*-MeLeu)			
	β	38.7, CH	1.80, m	*γ*, *δ*, CO			
	γ	25.0, CH_2	1.36, m 1.13, m	*α*, *β*, *γ′*, *δ*			
	γ′	15.3, CH_3	0.77, d (6.3)	*α*, *β*, *γ*			
	δ	11.6, CH_3	0.77, d (6.7)	*α*, *β*, *γ*			
	*N*H		7.73, d (9.0)	*α*, CO, CO(*N*-MeLeu)			

[a]Recorded at 125 MHz.
[b]Recorded at 500 MHz.

The 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide derivatives of acid hydrolyzate of **1** and the corresponding D-configuration and L-configuration standards were subjected to HPLC–MS analysis. The absolute configurations of the amino acid residues of **1** were confirmed by comparison of the HPLC retention times and molecular mass with the corresponding standards. The amino acid residues Hse, *N*-MeLeu and Ile units were determined to have the L-configuration, whereas the Ala unit was determined to have the D-configuration (Supplementary Table S1). Thus, the structure of trichopeptide A (**1**) was established (Figure 1).

Trichopeptide B (**2**) was obtained as a brown gum. It has a molecular formula of $C_{21}H_{31}N_3O_6$ (eight degrees of unsaturation) by HR–ESI–MS pseudomolecular ion peak ($[M+H]^+$ at *m/z* 422.2295, calcd for 422.2286). Analysis of its ^1H and ^{13}C NMR data (Table 1) revealed a structure similar to **1**, as one benzoyl connected to the Hse-Ala-*N*-MeLeu sequence. According to 113 of molecular mass less than **1**, the Ile residue was missing. The Ile residue is replaced by a hydroxyl (chemical shift of carboxylic carbon in *N*-MeLeu: δ_C 170.4 in **1**, δ_C 173.8 in **2**). Thus, the planar structure of **2** was proposed (Figure 2). The absolute configurations of the Hse and *N*-MeLeu residues in **2** were assigned as L-configuration and the Ala unit was assigned as D-configuration through Marfey's method (Supplementary Table S1). The structure of trichopeptide B (**2**) was established as shown (Figure 1).

Trichocyclodipeptide A (**3**) was isolated as white amorphous powder. It has the molecular formula of $C_{26}H_{40}N_4O_8$ (nine degrees of unsaturation) determined by HR–ESI–MS pseudomolecular ion peak ($[M+H]^+$ at *m/z* 537.2926, calcd for 537.2919). The ^{13}C NMR data of **3** showed 13 carbon resonances. In accordance with the molecular formula, we speculated that **3** had a completely symmetrical structure. The ^1H and ^{13}C NMR data (Table 2) of **3** revealed four methyl groups, ten methylenes including two *O*-methylene, four methines, four olefinic carbons and six carboxylic carbons. These data accounted for eight degrees of unsaturation. The structure of **3** was similar to eleutherazine B,[13] with a diketopiperazine skeleton. The difference between them was that the hydroxyl groups of eleutherazine B at C-10 and C-10′ are acetylated in **3**. Interpretation of the COSY of half side of the structure of **3** revealed two proton spin-systems of C-2-C-5 and C-9-C-10. The substructure of C-1-C-5 was deduced as Orn residue by analysis of its NMR data. The HMBC correlations

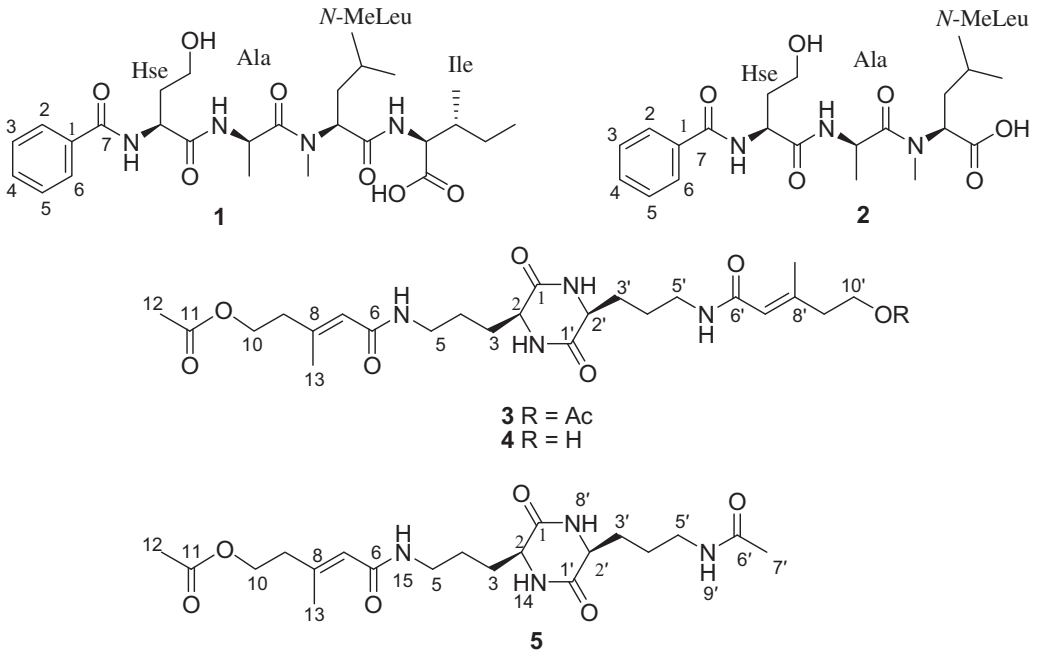

Figure 1 Structures of compounds **1**–**5**.

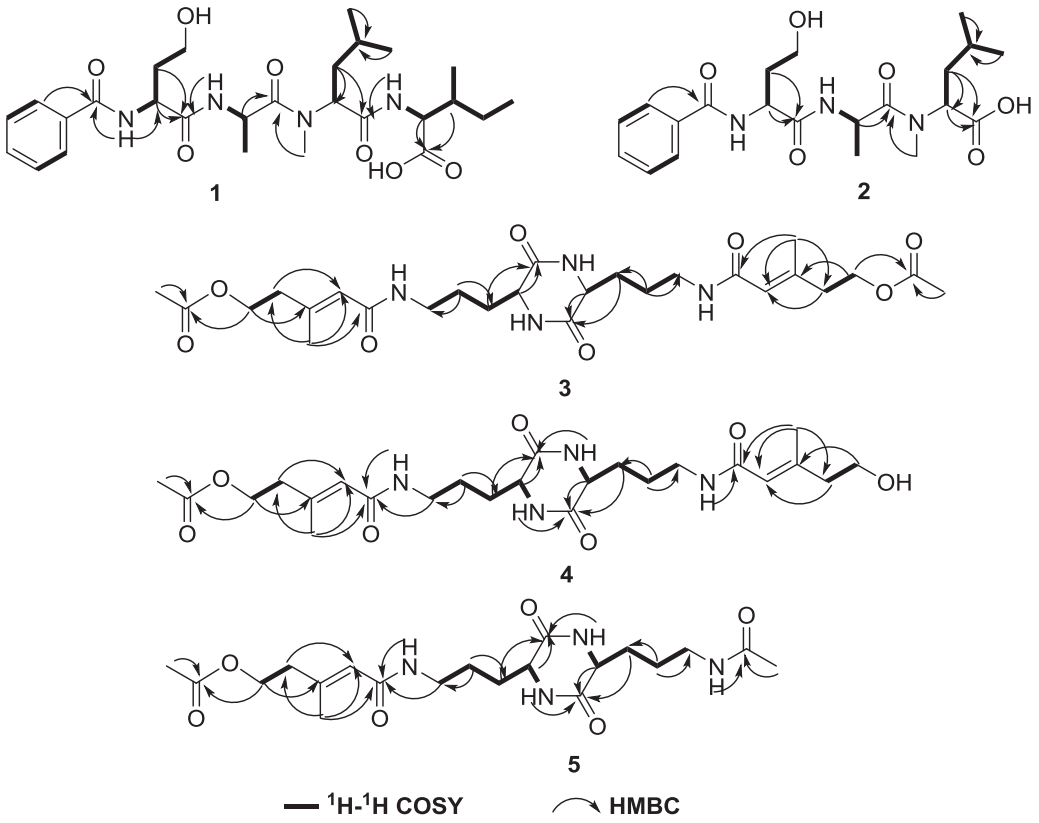

Figure 2 Key ^{1}H-^{1}H COSY, HMBC correlations of compounds **1**–**5**.

from olefinic proton H-7 (δ_H 5.68) to C-6 (δ_C 169.4) and C-8 (δ_C 150.7) indicated that C-6-C-8 was an*α*, *β*-unsaturated carbonyl. The correlations from H_2-9 (δ_H 2.39) and H_3-13 (δ_H 2.09) to C-7 (δ_C 121.2) indicated that the C-9 and C-13 (δ_c 18.4) were attached to C-8 and the moiety of C-6-C-10/C-13 was established. The correlations from H_2-5 (δ_H 3.19) and H-7 (δ_H 5.68) to C-6 (δ_C 169.4) indicated that C-5 (δ_C 39.5) and C-7 are connected by amide linkage. The chemical shift value of C-10 (δ_C 63.2) and the

HMBC correlations from H_3-12 (δ_H 1.98) to C-11 (δ_C 172.7) and H_2-10 (δ_H 4.17) to C-11 (δ_C 172.7) indicated that an acetyl group linked to C-10 through an oxygen atom. The chemical shift of C-2 (δ_C 55.7) and the HMBC correlations from H-2 (δ_H 3.96)/H-3 (δ_H 1.81) to C-1 (δ_C 170.4) indicated a lactone moiety. Comparing NMR data with leutherazine B indicated the existence of a cyclic dipeptide skeleton. Thus, the planar structure of **3** was established (Figure 2).

Table 2 NMR spectral data of compound 3 in CD_3OD

Position	δ_C[a] *type (δ in p.p.m.)*	δ_H[b] *(J in Hz)*	*HMBC (H→C)*
1, 1′	170.4, qC		
2, 2′	55.7, CH	3.96, t (5.4,5.3)	1, 1′, 3, 3′, 4, 4′
3, 3′	32.7, CH_2	1.81, m	1, 1′, 2, 2′, 4, 4′, 5, 5′
4, 4′	26.0, CH_2	1.57, m	2, 2′, 3, 3′, 5, 5′
5, 5′	39.5, CH_2	3.19, m	4, 4′, 3, 3′, 6, 6′
6, 6′	169.4, qC		
7, 7′	121.2, CH	5.68, s	6, 6′, 8, 8′, 9, 9′, 10, 10′, 13, 13′
8, 8′	150.7, qC		
9, 9′	40.4, CH_2	2.39, t (6.5,6.4)	7, 7′, 8, 8′, 10, 10′, 13, 13′
10, 10′	63.2, CH_2	4.17, t (6.6,6.5)	8, 8′, 9, 9′, 10, 10′, 11, 11′
11, 11′	172.7, qC		
12, 12′	20.8, CH_3	1.98, s	10, 10′, 11, 11′
13, 13′	18.4, CH_3	2.09, s	6, 6′, 7, 7′, 8, 8′, 9, 9′

[a]Recorded at 125 MHz.
[b]Recorded at 500 MHz.

Trichocyclodipeptide B (**4**) was obtained as white amorphous powder. It has the molecular formula of $C_{24}H_{38}N_4O_7$ (eight degrees of unsaturation) determined by HR–ESI–MS pseudomolecular ion peak $(M+H)^+$ at *m/z* 495.2816, calc dfor 495.2813). Most of the signals appeared in pairs, it was easy to deduce that the structure of **4** was similar to **3**. Compound **4** is 42 of molecular mass less than **3** and the chemical shifts of C-10′ shifted upfield (δ_H 4.17, δ_C 63.2 in **3**, δ_H 2.50, δ_C 59.1 in **4**), an acetyl group was missing in **4**, whereas rest of the substructure of **4** was corresponding to **3**.

Trichocyclodipeptides C (**5**) was obtained as white amorphous powder. It has the molecular formula of $C_{20}H_{32}N_4O_6$ (seven degrees of unsaturation) determined by HR–ESI–MS pseudomolecular ion peak ($[M+H]^+$ at *m/z* 425.2397, calcd for 425.2395). Analysis of its NMR data (Table 3) revealed the moiety of C-6′-C-10′/C-11′ in **4** was replaced by an acetyl group ($\delta_{H/C}$ 1.78/22.6; 169.0 in **5**). These results were confirmed by the HMBC correlations of from H_3-7′ (δ_H 1.78) and H_2-5′ (δ_H 3.00) to C-6′. Rest of the substructure of **5** was corresponding to **4**.

Table 3 NMR spectral data of compounds 4 and 5 in DMSO-d_6

	4			***5***		
Position	δ_C[a] *type (δ in p.p.m.)*	δ_H[b] *(J in Hz)*	*HMBC (H→C)*	δ_C[a] *type (δ in p.p.m.)*	δ_H[b] *(J in Hz)*	*HMBC (H→C)*
1	167.8, qC			167.8, qC		
2	53.9, CH	2.78, t (6.5, 6.4)	1	53.8, CH	3.80, t (6.5, 6.4)	1
3	30.9, CH_2	0.61, m	1, 2, 4, 5	30.8, CH_2	1.67, m	1, 2, 4, 5
4	24.8, CH_2	0.43, m	2, 3, 5	24.8, CH_2	1.44, m	2, 3, 5
5	38.0, CH_2	2.04, m	3, 4, 6	38.0, CH_2	3.05, m	3, 4, 6
6	165.7, qC			165.7, qC		
7	120.5, CH	4.63, s	6, 8, 9, 13	120.5, CH	5.64, s	6, 8, 9, 13
8	147.6, qC			147.5, qC		
9	38.8, CH_2	1.32, t (6.6, 6.5)	7, 9, 10, 13	38.8, CH_2	2.33, t (6.5, 6.4)	7, 9, 10, 13
10	61.6, CH_2	3.11, t (6.6, 6.5)	8, 9, 11	61.6, CH_2	4.12, t (6.4, 6.3)	8, 9, 11
11	170.3, qC			170.3, qC		
12	20.7, CH_3	0.98, s	10, 11	20.7, CH_3	1.99, s	10, 11
13	17.6, CH_3	1.07, s	6, 7, 8, 9	17.6, CH_3	2.08, s	6, 7, 8, 9
14	*N*H	7.12. s	1, 2	*N*H	8.13, s	1, 2
15	*N*H	6.80, t (5.1, 5.0)	5, 6	*N*H	7.81, t (5.2, 5.1)	5, 6
1′	167.8, qC			167.8, qC		
2′	53.8, CH	2.78, t (6.6, 6.5)	1′	53.9, CH	3.80, t (6.5, 6.4)	1′
3′	30.9, CH_2	0.67, m	1′, 2′, 4′, 5′	30.8, CH_2	1.63, m	1′, 2′, 4′, 5′
4′	24.9, CH_2	0.43, m	2′, 3′,5′	24.8, CH_2	1.44, m	2′, 3′, 5′
5′	38.0, CH	2.03, m	3′, 4′, 6′	38.3, CH_2	3.00, m	3′, 4′, 6′
6′	166.0, qC			169.0, qC		
7′	119.9, CH	4.61, s	6′, 8′, 9′, 11′	22.6, CH_3	1.78, s	6′
8′	149.3, qC			*N*H	8.13, s	1′, 2′
9′	43.6, CH_2	1.16, t (6.6, 6.5)	7′, 8′, 10′, 11′	*N*H	7.81, t (5.3,5.2)	5′, 6′
10′	59.1, CH_2	2.50, t (6.5, 6.4)	8′, 9′			
11′	17.9, CH_3	1.05, s	6′, 7′, 8′, 9′			
12′	*N*H	7.12, s	1′, 2′			
13′	*N*H	6.74, t (5.1, 5.0)	5′, 6′			

Abbreviation: DMSO, dimethyl sulfoxide.
[a]Recorded at 125 MHz.
[b]Recorded at 500 MHz.

The absolute configurations of the Orn residues in **3–5** were assigned as L-configuration through Marfey's method (Supplementary Table S1). Thus, the structures of **3–5** were established as shown (Figure 1).

Compounds **1–5** were tested for antimicrobial activity against the fungus *C. albicans* (CGMCC 2.2086), the Gram-positive bacterium *Staphylococcus aureus* (ATCC 6538), the Gram-negative bacterium *Escherichia coli* (ATCC 1.0090) and the *Bacillus subtilis* (ATCC 6663), respectively (Table 4). Compound **3** showed antifungal activity against *C. albicans* with the IC_{50} and MIC values of 22 and 90 μg ml^{-1}, respectively. The positive control amphotericin showed the IC_{50} and MIC values of 5.32 and 18.13 μg ml^{-1}, respectively. Compounds **2** and **5** showed the antibacterial activity against *S. aureus* (vancomycin: $IC_{50} = 0.08$, MIC = 0.54 μg ml^{-1}) with the IC_{50} values of 45 and 44 μg ml^{-1}, respectively. Compounds **3** and **5** showed the antibacterial activity against *B. subtilis* (streptomycin: $IC_{50} = 0.13$, MIC = 0.32 μg ml^{-1}) with the IC_{50} values of 46 and 45 μg ml^{-1}, respectively.

Compounds **1–5** were also tested for cytotoxic activity for two cancer cell lines including lung cell (A549) and leukemia cell (K562), evaluated with the CCK8 cell viability assay. Compound **4** has weak cytotoxicity against K562 with IC_{50} value of 132 μg ml^{-1}, whereas the positive control taxol showed IC_{50} value of 0.92 μg ml^{-1}.

Although linear peptides have been isolated frequently,[14–16] such as antibiotic-BK-230 from *Burkholderia glumae*, celenamides A and B from *Cliona celata*, compounds **1** and **2** distinctly differ from most of the known natural products. The presence of a D-Ala amino acid residue, only a few ones contained D-Ala amino acid residue, such as microcystin-LR from *Microcystis* spp., have been previously reported.[17] The compounds containing D-Ala amino acid residue were reported to possess growth inhibition bioactivity against some bacteria.[18] Diketopiperazines, such as eleutherazine B from the traditional Chinese medicine plant *Acanthopanax senticosus*, metachelins A and B from the insect-pathogenic fungus *Metarhizium robertsii* were discovered recently,[19] and alternarizines A and B from an endophytic fungus *Alternaria alternata* and talarazines A–E, from an Australian mud dauber wasp-associated fungus, *Talaromyces* sp.[20,21]. However, little antimicrobial activity was reported, the new structures **3–5** showed the moderate antimicrobial activities.

MATERIALS AND METHODS

General experimental procedures

Optical rotations were measured on an Anton Paar MCP200 polarimeter (Graz, Austria). ^{1}H and ^{13}C NMR data were acquired with Bruker Avance-500 spectrometers (Rheinstetten, Germany). The HSQC and HMBC experiments were optimized for 125.0 and 8.0 Hz, respectively. HR–ESI–MS and HPLC–ESI–MS data were recorded on an Accurate-Mass-Q-TOF LC/MS 6520 instrument (Santa Clara, CA, USA) in positive ion mode. HPLC data were obtained with a Waters 2695 instrument (Milford, MA, USA). Preparative HPLC was performed on an Agilent 1200 HPLC system using a C_{18} column (7.8 × 300 mm, Waters, 7 μm; detector: UV) with a flow rate of 2.2 ml min^{-1}. The absorbance of contents in the 96-well clear plate was detected by a SpectraMax Paradigm microplate reader (Sunnyvale, CA, USA).

Fungal material

The strain of *S. trichophoricola* was isolated from a soil sample surrounding the fruiting body of *O. sinensis* collected in Maqin Country, Qinghai Province, People's Republic of China, in 2014. The isolated strain was identified by morphology and sequence analysis (Genbank Accession Number KY750315) of the rDNA internal transcribed spacer (ITS) region. The strain was firstly cultivated on culture dish of potato-dextrose agar at 25 °C for 10 days. Then the agar was cut into grain size to inoculate in two conical flasks (500 ml) that each containing 300 ml autoclave sterilized potato-dextrose broth. The flasks containing inoculated potato-dextrose broth were cultivated at 25 °C on a rotary shaker at 170 rpm for 5 days. Thirty 500 ml Fernbach flasks, each containing 80 g of rice and 100 ml of distilled water, were autoclaved at 120 °C for 30 min, in which the fermentation proceeded. After cooling to room temperature, each of the flasks was inoculated with 10 ml of the spore inoculum and cultured at 25 °C for 30 days.

Extraction and isolation

The ferment culture was extracted with EtOAc (three times, each 6 l) and vacuum-dried to afford the crude extract (~10 g). The extract was fractionated by silica gel vacuum liquid chromatography (VLC) in petroleum ether-Acetone-MeOH gradient elution (Supplementary Figure S1). The fraction (2 g) eluted with 10% MeOH was then loaded on a silica gel column (2.5 × 45 cm) eluted with CH_2Cl_2-MeOH. The fraction (538 mg) eluted from 20:1 CH_2Cl_2:MeOH was isolated by Sephadex LH-20 column chromatography then eluted with MeOH, the subfractions were selected and purified by RP HPLC (Waters Symmetry Prep C_{18} column; 7 μm; 7.8 × 300 mm; 60–70% MeOH in H_2O with 0.1% HCOOH for 45 min; 2.2 ml min^{-1}) to afford **1** (10.0 mg, t_R19 min) and **2** (6.0 mg, t_R 17.00 min) (Supplementary Figure S2). The fraction (368 mg) eluted from 10:1 CH_2Cl_2:MeOH was isolated by Sephadex LH-20 column chromatography eluted in MeOH. The subfractions were purified by RP HPLC (Waters Symmetry Prep C_{18} column; 7 μm; 7.8 × 300 mm; 40–60% MeOH in H_2O with 0.1% HCOOH for 50 min; 2.2 ml min^{-1}) to afford **3** (10.0 mg, t_R 27.20 min), **4** (8.0 mg, t_R 20.50 min) and **5** (6.0 mg, t_R 23.00 min) (Supplementary Figure S2).

Compounds characterization

*Trichopeptide A (**1**)*: Brown gum; $[\alpha]_D$ −9.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 224 nm (log ε) (3.98); NMR data (500 MHz, $CDCl_3$) see Table 1; HR–ESI–MS *m/z* 535.3124 $[M+H]^+$ (calcd for $C_{27}H_{43}N_4O_7$ 535.3126).

*Trichopeptide B (**2**)*: Brown gum; $[\alpha]_D$ −48.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 210 nm (log ε) (3.86); NMR data (500 MHz, Acetone-d_6)

Table 4 Antimicrobial activities of compounds 1–5

	C. albicans		*S. aureus*		*B. subtilis*	
Compound	*IC_{50} (μg ml^{-1})*	*MIC (μg ml^{-1})*	*IC_{50} (μg ml^{-1})*	*MIC (μg ml^{-1})*	*IC_{50} (μg ml^{-1})*	*MIC (μg ml^{-1})*
1	59	>100	57	>100	77	>100
2	52	>100	45	>100	52	>100
3	22	90	51	>100	46	>100
4	66	>100	54	>100	68	>100
5	62	>100	44	>100	45	>100
Amphotericin	5.32	18.13	—	—	—	—
Vancomycin	—	—	0.08	0.54	—	—
Streptomycin	—	—	—	—	0.13	0.32

see Table 1; HR–ESI–MS *m/z* 422.2295 $[M+H]^+$ (calcd for $C_{21}H_{32}N_3O_6$ 422.2286).

Trichocyclodipeptide A (***3***): White powder; $[\alpha]_D$ −22.0 (*c* 0.05, MeOH); UV (MeOH) λ_{max} 222 nm (log ε) (3.76); NMR data (500 MHz, CD_3OD) see Table 2; HR–ESI–MS *m/z* 537.2926 $[M+H]^+$ (calcd for $C_{26}H_{41}N_4O_8$ 537.2919).

Trichocyclodipeptide B (***4***): White powder; $[\alpha]_D$ −14.0 (*c* 0.05, MeOH); UV (MeOH) λ_{max} 215 nm (log ε) (3.83); NMR data (500 MHz, dimethyl sulfoxide-d_6) see Table 3; HR–ESI–MS *m/z* 495.2816 $[M+H]^+$ (calcd for $C_{24}H_{39}N_4O_7$ 495.2813).

Trichocyclodipeptide C (***5***): White powder; $[\alpha]_D$ −18.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 210 nm (log ε) (3.67); NMR data (500 MHz, dimethyl sulfoxide-d_6) see Table 3; HR–ESI–MS *m/z* 425.2397 $[M+H]^+$ (calcd for $C_{20}H_{33}N_4O_6$ 425.2395).

Absolute configuration

Compounds **1–5** (1 mg each) with 6 N HCl (2.0 ml) were heated at 115 °C for 10 h.[22] Then the hydrolyzates were placed in a 1 ml reaction vial added 60 µl 1.0 N $NaHCO_3$ and 150 µl 1% solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide in acetone. The vials were heated at 45 °C for 2 h, after cooling to room temperature, adding 30 µl 2.0 N HCl. The corresponding L- and D- standards were derivatized in the same was. The derivatives of the hydrolyzates and the standards were subjected to HPLC–MS analysis (Hypersil GOLD C_{18} column; 5 µm, 4.6 × 250 mm; 1.0 ml min^{-1}) at 30 °C in gradient program: linear gradient 15–45% MeCN in H_2O with 0.1% HCOOH for 50 min at 340 nm UV detection. The retention time data were recorded (Supplementary Table S1).

Antimicrobial assay

The antimicrobial assay was following the recommendations from the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards (NCCLS))[23,24] and conducted in triplicate. The fungal strain, *C. albicans* (CGMCC 2.2086), was grown in potato-dextrose broth, whereas the bacterial strain, *S. aureus* (ATCC 6538), *B. subtilis* (ATCC 6663) and *E. coli* (ATCC 1.0090) were grown in lysogeny broth. The targeted microbe was cultivated in broth at 25 °C for 48 h (fungi) and at 37 °C for 24 h (bacteria), till the final suspension reached 10^6 cells ml^{-1}. Test samples (10 mg ml^{-1} as stock solution in dimethyl sulfoxide and serial dilutions) were transferred to a 96-well clear plate in triplicate, and the suspension of the test organism was added to each well, achieving a final volume of 100 µl (amphotericin, vancomycin, streptomycin and kanamycin were used as the positive control). After incubating at 25 °C for 48 h (fungi) and at 37 °C for 24 h (bacteria), the absorbance was detected with a microplate reader under 595 nm. The inhibition was calculated and plotted vs test concentrations to afford the IC_{50} and MIC.

Cytotoxicity bioassay

Cytotoxic activity for cancer cell lines including A549 and K562 cell lines was evaluated with the CCK8 cell viability assay.[23] After treating cells with the compounds tested (in dimethyl sulfoxide) for 48 h in 96-well plates, adding 3 µl of CCK8 medium solution to each well, then culturing the tumor cells at 37 °C in a humidified atmosphere of 5% CO_2 air for 4 h. The plate was read by microplate reader under 450 nm. The inhibition was calculated and plotted vs test concentrations to afford the IC_{50} in triplicate.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Dreyfuss, M. M. & Chapela, I. H. Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals. *Biotechnology* **26**, 49–80 (1994).
2 Tan, R. X. & Zou, W. X. Endophytes: a rich source of functional metabolites. *Nat. Prod. Rep.* **18**, 448–459 (2001).
3 Gloer, J. B. Antiinsectan natural products from fungal sclerotia. *Acc. Chem. Res.* **28**, 343–350 (1995).
4 Keller, N. P. & Wiemann, P. Strategies for mining fungal natural products. *J. Ind. Microbiol. Biotechnol.* **41**, 301–313 (2014).
5 Guo, H. *et al.* Bioactive *p*-terphenyl derivatives from a *Cordyceps*-colonizing isolate of *Gliocladium* sp. *J. Nat. Prod.* **70**, 1519–1521 (2007).
6 Li, E. *et al.* A spiro [chroman-3,7′- isochromene]-4,6′(8′H)-dione from the *Cordyceps*-colonizing fungus *Fimetariella* sp. *Org. Lett.* **14**, 3320–3323 (2012).
7 Deng, L., Niu, S., Liu, X., Che, Y. & Li, E. Coniochaetones E-I, new 4*H*-chromen-4-one derivatives from the *Cordyceps*-colonizing fungus *Fimetariella* sp. *Fitoterapia* **89**, 8–14 (2013).
8 Lin, J. *et al.* Polyketides from the ascomycete fungus *Leptosphaeria* sp. *J. Nat. Prod.* **73**, 905–910 (2010).
9 Ahonsi, M. O., Maurhofer, M., Boss, D. & Defago, G. Relationship between aggressiveness of *Stagonospora* sp. isolates on field and hedge bindweeds, and in vitro production of fungal metabolites cercosporin, elsinochrome A and leptosphaerodione. *Eur. J. Plant Pathol.* **111**, 203–215 (2005).
10 Evidente, A., Cimmino, A., Berestetskiy, A., Andolfi, A. & Motta, A. Stagonolides G-I and modiolide A, nonenolides produced by *Stagonospora cirsii*, a potential mycoherbicide for *Cirsium arvense*. *J. Nat. Prod.* **71**, 1897–1901 (2008).
11 Schreiber, D. *et al.* 3'-Demethyldihydromaldoxin and dihydromaldoxin, two anti-inflammtory diaryl ethers from a *Staganospora* species. *J. Antibiot.* **65**, 473–477 (2012).
12 Harrison, P. H. M. *et al.* The biosynthesis of pramanicin in *Stagonospora* sp. ATCC 74235: a modified acyltetramic acid. *Perkin Trans 1.* **24**, 4390–4402 (2000).
13 Li, Z. F., Xu, N., Feng, B. M., Zhang, Q. H. & Pei, Y. H. Two diketopiperazines from *Acanthopanax senticosus* harms. *J. Asian Nat. Prod. Res.* **12**, 51–55 (2010).
14 Mitchell, R. E., Greenwood, D. R. & Sarojini, V. An antibacterial pyrazole derivative from *Burkholderia glumae*, a bacterial pathogen of rice. *Phytochemistry* **69**, 2704–2707 (2008).
15 Stonard, R. J. & Andersen, R. J. Celenamides A and B, linear peptide alkaloids from the sponge *Cliona celata*. *J. Org. Chem.* **45**, 3687–3691 (1980).
16 Singh, S. B. *et al.* Integramides A and B, two novel non-ribosomal linear peptides containing nine c^{α}-methyl amino acids produced by fungal fermentations that are inhibitors of HIV-1 integrase. *Org. Lett.* **4**, 1431–1434 (2002).
17 Choi, B. W. *et al.* Isolation of linear peptides related to the hepatotoxins nodularin and microcystins. *Tetrahedron Lett.* **34**, 7881–7884 (1993).
18 Neuhaus, F. C., Goyer, S. & Neuhaus, D. W. Growth inhibition of *Escherichia coli* W by D-norvalyl-D-alanine: an analogue of D-alanine in position 4 of the peptide subunit of peptidoglycan. *Antimicrob. Agents Chemother.* **11**, 638–644 (1977).
19 Krasnoff, S. B., Keresztes, I., Donzelli, B. G. G. & Gibson, D. M. Metachelins, Mannosylated and *N*-Oxidized coprogen-type siderophores from *Metarhizium robertsii*. *J. Nat. Prod.* **77**, 1685–1692 (2014).
20 Guo, D. L. *et al.* Two new diketopiperazines and a new glucosyl sesterterpene from *Alternaria alternata*, an endophytic fungi from *Ceratostigma griffithii*. *Phytochem. Lett.* **14**, 260–264 (2015).
21 Kalansuriya, P., Quezada, M., Espósito, B. P. & Capon, R. J. Talarazines A-E: noncytotoxic iron(III) chelators from an Australian mud dauber wasp-associated fungu *Talaromyces* sp. (CMB-W045). *J. Nat. Prod.* **80**, 609–615 (2017).
22 Vijayasarathy, S. *et al.* C_3 and 2D C_3 marfey's methods for amino acid analysis in natural products. *J. Nat. Prod.* **79**, 421–427 (2016).
23 Salem, M. S. & Ali, M. A. Novel pyrazolo[3,4-*b*] pyridine derivatives: synthesis, characterization, antimicrobial and antiproliferative profile. *Biol. Pharm. Bull.* **39**, 473–483 (2016).
24 Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Testing for Bacteria that Grew Aerobically*, Approved Standard M7-A10; (Clinical and Laboratory Standards Institute, Wayne, PA, 2009).

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