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Two New Diketopiperazine Alkaloids from a Deep-soil Derived

Fungus Penicillium simplicissimum GZWMJZ-1612

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Abstract: Two new diketopiperazine alkaloids, namely simplicamides A (1) and B (2), were isolated from the deep-soil derived fungus *Penicillium simplicissimum* GZWMJZ-1612. The structures of these two compounds were determined by NMR, HRESIMS, ECD calculation, and Marfey's method. Compound 1 exhibited potent antioxidant capacity of ORAC and DPPH scavenging ability. Compound 2 displayed significant ORAC scavenging ability and α -glucosidase inhibitory activity.

Keywords: Deep-soil; *Penicillium simplicissimum*; diketopiperazine alkaloids; antioxidant capacity; α -gluco-sidase. © 2025 ACG Publications. All rights reserved.

1. Introduction

Microbial secondary metabolites are the major source for the discovery of new active compounds [1-2]. To date, more than twenty thousand secondary metabolites with biological activities have been isolated from microorganisms [3-4]. Among them, the diversity of secondary metabolites from *Penicillium* provides abundant resources for the discovery of structurally new active natural products [5-6]. For example, Dai et al. [7] isolated indole diterpene compounds from marine fungus *Penicillium* sp. ZYX-Z-143 with antidiabetic and anti-inflammatory activities. Kaliaperumal et al. [8] isolated averufin, which exhibits strong anticancer activity toward myeloid leukemia from the plant endophyte *Penicillium verruculosum*. Weng et al.[9] isolated polyketones from the endophyte *Penicillium* sp.YT2019-3321 of *Lonicera Japonica* with good cytotoxic activity. *Penicillium simplicissimum*, an important member of

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the *Penicillium* genus, has garnered significant attention in the field of natural product research due to its potential to produce diverse secondary metabolites. According to literature reports, *P. simplicissimum* strains are a rich source of various bioactive compounds. For instance, Wang et al. [10] isolated two novel polyketide-peptide hybrid alkaloids from the soil fungus *P. simplicissimum* JXCC5. They found that the compound penisimplicins A demonstrated significant acetylcholinesterase inhibitory activity. Yang et al. [11] isolated ten new compounds from the marine fungus *P. simplicissimum*. Compounds Penisimplinoids G and K exhibited anti - inflammatory and notable angiogenesis-promoting activities. Dai et al. [12] isolated seven new quinolones from the soil fungus *P. simplicissimum*. Among them, Pesimquinolones A, E, G, and H showed promising inhibitory effects on nitric oxide (NO) production. All these findings suggest that the secondary metabolites of *P. simplicissimum* contain abundant chemical resources, making it worthy of further exploration. In this study, we investigated the fermentation process, chemical composition, and biological activity of a deep-soil derived fungus *Penicillium simplicissimum* GZWMJZ-1612. Through this comprehensive analysis, two new diketopiperazine alkaloids (compounds **1** and **2**) were successfully isolated. These compounds demonstrated varying degrees of antioxidant and α -glucosidase inhibitory activities.

2. Materials and Methods

2.1. Fungal Material

The fungus strain of *P. simplicissimum* GZWMJZ-1612 was isolated from a soil sample at a depth of 297 meters in Bijie, Guizhou Province, China. Through ITS gene sequencing, the obtained sequences were compared with the NCBI-BLAST database. The results demonstrated a 100% sequence identity with *P. simplicissimum* clone EF_529. Consequently, the strain was identified as *P. simplicissimum* GZWMJZ-1612 (GenBank No. PV069431) and preserved at -80°C in the Natural Products Research Center of Guizhou Province.

2.2 Cultivation and Extraction

The strain was initially activated on potato dextrose agar PDA at 28°C for three days. It was then transferred to a rice medium totaling 10 kg, and distributed into 200 bags (polypropylene plastic bag with a diameter of 89 mm, length of 280 mm, thickness of 0.055 mm), each containing 40 mL of water. The culture was maintained at room temperature for 30 days. Subsequently, the extract was obtained through three successive ethyl acetate (EtOAc) extractions, with a solvent volume three times the column volume for each extraction. The combined extracts were vacuum-dried, yielding 118 g of extract.

2.3. Purification

The crude extract (118 g) was fractionated using 5 times the volume 200-300 mesh silica gel column (diameter 80 mm, length 610 mm) chromatography and eluted with a petroleum ether (PE)/EtOAc (100:0 10:1 2:1 1:1 1:5 0:100) gradient, yielding 13 fractions (Fr.1 - Fr.13). Fr. 9 (1.2 g) was further purified by Sephadex LH-20 column chromatography using dichloromethane (CH₂Cl₂)/methanol (MeOH) (1 : 1) as the eluent, resulting in four subfractions (Fr.9-1 - Fr.9-4). Fr. 9-3 (170 mg) was subjected to Toyopearl HW-40 column chromatography with CH₂Cl₂-MeOH (1 : 1), yielding seven additional subfractions (Fr.9-3-1 - Fr.9-3-7). Fr. 9-3-4 (64 mg) was further purified by HPLC on a YMC-Pack ODS-A column (10 × 250 mm, 5 µm) using 60% MeOH - 40% distilled water (H₂O) (0.5‰ trifluoroacetic acid (TFA)) at a flow rate of 4 mL/min, leading to the isolation of compound 1 (26.3 mg, t_R 19.4 min). Additionally, Fr.7 (3.1 g) was separated by Sephadex LH-20 column eluted with CH₂Cl₂/MeOH (1 : 1) to afford nine subfractions (Fr.7-1 - Fr.7-9). Fr.7-7 (1.22 g) was subjected to *Toyopearl* HW-40 chromatography eluted with CH₂Cl₂-MeOH (1 : 1) to afford nine additional subfractions (*Fr*.7-7-1 – *Fr*.7-7-9). *Fr*. 7-7-6 (458 mg) was further purified by using a *YMC-Pack ODS-A* column (10 × 250 mm, 5 µm) eluted with 60% MeOH - 40% H₂O (0.5‰ TFA) at a flow rate of 4 mL/min, to yield compound **2** (14.2 mg, t_R 21.0 min).



Figure 1. Structure of compounds 1 and 2

2.4. Spectral Data

Simplicamide A (1): A brown solid; $[\alpha]_{D}^{20}$ +212.1 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.1), 255 (4.3), 282 (3.7), 321 (3.4) nm; IR (KBr) v_{max} 3459, 2974, 2932, 1703, 1657, 1648, 1469, 1398, 1340, 1261, 1156, 949, 887, 761, 689 cm⁻¹; HRESIMS at *m*/*z* 448.2213 [*M* + H]⁺ (calcd for C₂₆H₃₀N₃O₄ 448.2231); ¹H and ¹³C NMR (600 and 150 MHz, CDCl₃) data in Table 1.

Simplicamide B (2): A brown solid; $[\alpha]_{D}^{20}$ +290.5 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.8), 250 (4.6), 280 (4.1), 325 (3.7) nm; ECD (1.12 *m*M, MeOH) λ_{max} ($\Delta \varepsilon$) 236 (-22.9), 260 (+42.0); IR (KBr) ν_{max} 3321, 2974, 2929, 1703, 1670, 1644, 1453, 1384, 1258, 1154, 950, 900, 732 cm⁻¹; HRESIMS at *m*/*z* 446.2065 [*M* + H]⁺ (calcd for C₂₆H₂₈N₃O₄ 446.2074), *m*/*z* 468.1884 [*M* + Na]⁺ (calcd for C₂₆H₂₇N₃O₄Na 468.1894); ¹H and ¹³C NMR (600 and 150 MHz, DMSO-*d*₆) data in Table 1.

2.5. Marfey's Method

The configuration of the C-17 chiral center in compound **1** was determined by Marfey's method [13]. HPLC analysis was conducted on a C18 column (*YMC-Pack ODS-A*, 4.6×250 mm, 5 µm) with a flow rate of 1 mL/min. The mobile phase consisted of a linear gradient of acetonitrile (MeCN) (A) - H₂O (TFA 0.5‰) (B), with the following gradient elution conditions: 0 - 40 min, 30 - 60% (A); 40 - 45 min, 100% (A). The retention times for compound **1**, L-proline, and D-proline derivatives were 19.4 min, 19.4 min, and 22.9 min, respectively.



Figure 2. The key ¹H-¹H COSY, HMBC, NOESY correlations and the structures of compounds 1-2

2.6. Biological Activity Assays

The oxygen radical absorbance capacity assay (ORAC) [14], the DPPH radical scavenging assay (DPPH) [15] and the α -glucosidase inhibitory activity assay [16] were conducted according to the methods reported in the literature.

3. Results and Discussion

3.1. Structure Elucidation

Compound 1 was isolated as a brown solid, and the formula was determined to be $C_{26}H_{29}N_{3}O_{4}$ based on HRESIMS at m/z 448.2213[M + H]⁺ (calcd for C₂₆H₃₀N₃O₄ 448.2231). The IR spectrum at 1657 and 1648 cm⁻¹ suggested the presence of diketopiperazine part in 1 [14]. The 1D (Table 1) and HSQC NMR data displayed one NH ($\delta_{\rm H}$ 8.29), four methyl groups ($\delta_{\rm H/C}$ 1.72/24.7, 1.40/27.7, 1.40/27.9, 1.40/28.0), five sp³-methylenes ($\delta_{H/C}$ 0.08&1.83/29.5, 1.09&1.48/20.9, 3.08&3.46/45.2, 2.95&3.70/27.5, 3.49&5.56/53.9), two sp³-methines ($\delta_{H/C}$ 3.72/58.6, 4.27/61.6), four sp²-methines ($\delta_{H/C}$ 5.58/129.6, 6.44/123.3, 6.71/98.0, 7.06/116.3), two sp³-nonprotonated carbons ($\delta_{\rm C}$ 48.3, 76.1), six nonprotonated olefinic carbons ($\delta_{\rm C}$ 103.0, 116.4, 121.8, 135.6, 138.0, 149.8), and three carbonyl groups ($\delta_{\rm C}$ 163.7, 165.9, 209.7) (Table 1). These spectroscopic data indicate that the planar structure of compound 1 closely resembles that of carneamide C [17]. However, a notable distinction is observed in the presence of C-20 methylene and C-21 ketocarbonyl instead of the unsaturated olefin of $\Delta^{20,21}$ in carneamide C. The HMBC correlations from H₂-20 ($\delta_{\rm H}$ 3.49/5.56) to C-11 ($\delta_{\rm C}$ 61.6), C-21 ($\delta_{\rm C}$ 209.7), C-22 ($\delta_{\rm C}$ 48.3) and C-18 ($\delta_{\rm C}$ 165.9), from H₃-23 ($\delta_{\rm H}$ 1.40) and H₃-24 ($\delta_{\rm H}$ 1.72) to C-22, C-21, and C-2 ($\delta_{\rm C}$ 138.0) confirmed this structural fragment. Additionally, the NOESY correlation between H-11 ($\delta_{\rm H}$ 4.27) and H-17 ($\delta_{\rm H}$ 3.72) indicated the cis-configuration of the diketopiperazine ring. Meanwhile, the result of Marfey's analysis of 1 revealed the presence of L-proline. Therefore, the stereogenic centers of 1 were determined as 11S and 17S and named simplicamide A (Figure 1).

Compound 2 was isolated as a brown solid. The molecular formula was determined to be $C_{26}H_{27}N_{3}O_{4}$ based on HRESIMS at m/z 446.2065 $[M + H]^{+}$ (calcd for $C_{26}H_{28}N_{3}O_{4}$ 446.2074). The IR spectrum at 1670 and 1644 cm⁻¹ suggested the presence of diketopiperazine segment in 2 [17]. The ¹H, ¹³C, and HSQC NMR data (Table 1) displayed one NH ($\delta_{\rm H}$ 10.91), four methyl groups ($\delta_{\rm H/C}$ 1.63/24.3, 1.30/27.4, 1.32/27.4, 1.33/27.4), four sp³-methylenes ($\delta_{\rm H/C}$ 2.02&2.41/27.1, 2.77& 3.37/26.7, 3.33&3.70/45.2, 3.60&5.38/53.9), one sp³-methine ($\delta_{H/C}$ 4.54/62.2), five sp²-methines ($\delta_{H/C}$ 5.62/129.1, 6.42/123.3, 5.63/118.5, 6.60/97.7, 6.86/114.9), two sp³-nonprotonated carbons ($\delta_{\rm C}$ 48.1, 75.3), seven nonprotonated olefinic carbons ($\delta_{\rm C}$ 101.4, 115.0, 121.8, 131.6, 135.9, 138.7, 148.4), three carbonyl groups ($\delta_{\rm C}$ 155.4, 161.6, 210.2) (Table 1). The comprehensive analysis of the ¹H, ¹³C and HSQC NMR spectra indicates that the indole moiety and the diketopiperazine segment of 2 exhibit significant structural similarities to those of compound 1. However, a notable distinction is observed in 2, which possesses two olefinic carbons: an unprotonated olefinic carbon at C-17 ($\delta_{\rm C}$ 131.6) and a sp²-hybridized methylene group at C-16 ($\delta_{H/C}$ 5.63/118.5). The COSY correlations of H₂-15 (δ_{H} 2.02&2.41)/H₂-14 (δ_{H} 3.33&3.70/H-16 ($\delta_{\rm H}$ 5.63), along with the HMBC correlations from H₂-14 to C-12 ($\delta_{\rm C}$ 161.6)/C-17 ($\delta_{\rm C}$ 131.6), confirmed the double bond substitution at C-16 and C-17. The configuration was further confirmed by calculations of the electronic circular dichroism (ECD) of 2 using the time-dependent density

functional theory (TD-DFT) at the B3LYP/6-31G(d) level, and the detailed calculation process is provided in the supporting information. The results showed that the measured ECD curve matched well with the calculated ECD for S-2 and was opposite of that of R-2 (Figure 3). Therefore, the structure of 2 was defined and named simplicamide B (Figure 1).

Position	1 (CDCl ₃)		2 (DMSO- <i>d</i> ₆)	
	$\delta_{ m H}$	$\delta_{ m C}$	$\delta_{ m H}$	$\delta_{ m C}$
1(NH)	8.29 (1H, s)		10.91 (1H, s)	
2		138.0, C		138.7, C
3		103.0, C		101.4, C
4	7.06 (1H, s)	116.3, CH	6.86 (1H, s)	114.9, CH
5		116.4, C		115.0, C
6		149.8, C		148.4, C
7	6.71 (1H, s)	98.0, CH	6.60 (1H, s)	97.7, CH
8		135.6, C		135.9, C
9		121.8, C		121.8, C
10	3.70 (1H, m) 2.95 (1H, dd, 15.9, 6.0)	27.5, CH ₂	3.37 (1H, m) 2.77 (1H, dd, 15.7, 6.3)	26.7, CH ₂
11	4.27 (1H, d, 6.0)	61.6, CH	4.54 (1H, d, 6.3)	62.2, CH
12		163.7, C		161.6, C
14	3.46 (1H, dt, 12.0, 7.6) 3.08 (1H, td, 11.2, 4.2)	45.2, CH ₂	3.70 (1H, td, 11.8, 7.4) 3.33 (1H, dt, 11.8, 6.0)	45.2, CH ₂
15	1.48 (1H, m) 1.09 (1H, m)	20.9, CH ₂	2.41 (1H, m) 2.02 (1H, m)	27.1, CH ₂
16	1.83 (1H, dt, 11.9, 6.6) 0.08 (1H, m)	29.5, CH ₂	5.63 (1H, t, 3.0)	118.5, CH
17	3.72 (1H, m)	58.6, CH		131.6, C
18		165.9, C		155.4, C
20	5.56 (1H, d, 16.6) 3.49 (1H, d, 16.6)	53.9, CH ₂	5.38 (1H, d, 16.6) 3.60 (1H, d, 16.6)	53.9, CH ₂
21		209.7, C		210.2, C
22		48.3, C		48.1, C
23	1.40 (3H, s)	27.9, CH ₃	1.30 (3H, s)	27.4, CH ₃
24	1.72 (3H, s)	24.7, CH ₃	1.63 (3H, s)	24.3, CH ₃
25	6.44 (1H, d, 9.6)	123.3, CH	6.42 (1H, d, 9.6)	123.3, CH
26	5.58 (1H, d, 9.6)	129.6, CH	5.62 (1H, d, 9.6)	129.1, CH
27		76.1, C		75.3, C
28	1.40 (3H, s)	27.7, CH ₃	1.32 (3H, s)	27.4, CH ₃
29	1.40 (3H, s)	28.0, CH ₃	1.33 (3H, s)	27.4, CH ₃

Table 1. The ¹H and ¹³C (600 and 150 MHz) NMR data of compounds 1 and 2 (δ in ppm, J in Hz)

Two new diketopiperazine alkaloids from a deep-soil derived



Figure 3. Experimental and calculated ECD curves of compound 2

3.2. Bioactivity

In this study, the antioxidant and α -glucosidase inhibitory activities of two isolated compounds were evaluated. The antioxidant activity was assessed using ORAC and DPPH. The results indicated that compounds **1** and **2** exhibited superior oxygen radical scavenging ability compared to the positive control Trolox, with ORAC values of 2.10 ± 0.02 and $2.22 \pm 0.01 \,\mu\text{M}$ TE/ μ M, respectively. Compound **1** displayed DPPH scavenging ability approaching that of *L*-ascorbic acid (VC) with an IC₅₀ value of $31.72 \pm 0.86 \,\mu\text{M}$ (VC 18.78 $\pm 0.08 \,\mu\text{M}$). Additionally, the PNPG method was utilized to assess the inhibitory activity against α -glucosidase. Compound **2** showed more potent inhibitory activity than acarbose, with an IC₅₀ value of $131.74 \pm 0.95 \,\mu\text{M}$ (acarbose $280.82 \pm 2.17 \,\mu\text{M}$) (table 2). These findings suggest that these compounds possess significant antioxidant and α -glucosidase inhibitory activities.

Table 2. The antioxidant and a-grycosidase activities of compounds 1-2					
Compound	ORAC (µM TE/µM)	DPPH (IC50, µM)	α -glucosidase (IC ₅₀ , μ M)		
1	2.10 ± 0.02	31.72 ± 0.86	>500		
2	2.22 ± 0.01	>100	131.74 ± 0.95		
VC	-	18.78 ± 0.08	-		
Acarbose	-	-	280.82 ± 2.17		
- not tested					

Table 2. The antioxidant and α -glycosidase activities of compounds 1-2

4. Conclusion

In this study, two new indolepiperazine alkaloids were isolated from the secondary metabolites of *P. simplicissimum* GZWMJZ-1612. Biological evaluations revealed that compounds **1** and **2** exhibited significant ORAC absorbance activity, approximately twice as effective as Trolox. Compound **1** demonstrated potent DPPH scavenging activity. Compound **2** showed twice the inhibitory activity against α -glucosidase compared to the positive control acarbose. In conclusion, this study demonstrates that compounds of indole diketopiperazine exhibit promising potential for antioxidant and α -glucosidase inhibitory activities. Moreover, the investigation of secondary metabolites from deep-soil derived fungus represents a significant avenue for the discovery of new bioactive natural products.

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Supporting Information

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

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