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Diketopiperazines and 2*H*-pyran-2-ones with antioxidant activity from the rice fermented with *Aspergillus luchuensis*

Shanshan Sun[a,b], Ke Ma[b,c], Qiaoqiao Tao[b,c], Junjie Han[b], Li Bao[b], Li Liu[b,c], Hongwei Liu[b,c,*]

[a] School of Life Sciences, University of Science and Technology of China, Hefei 230022, PR China
[b] State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, No. 1 Beichenxi Road, Chaoyang District, Beijing 100101, PR China
[c] Savaid Medical School, University of Chinese Academy of Sciences, Beijing 100049, PR China

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ABSTRACT

Aspergillus luchuensis is widely used as a starter of saccharification in the *koji* industry, but no secondary metabolites have been reported from this fungus. Herein, we report the isolation and identification of four new diketopiperazine derivatives (**1–4**), one new methyl 4-(3-acetyl-2, 6-dihydroxyphenyl)-2-methoxybutanoate (**5**), and six known compounds (**6–11**) from the rice *koji* of *A. luchuensis*. The structures of **1–5** were determined by extensive spectral analysis including 1D and 2D NMR, HRESIMS, and CD, and ECD calculation. In antioxidant assays, compound **10** displayed moderate DPPH scavenging activity with an EC_{50} value of 60.8 μM; compounds **1–4, 10** and **11** showed reducing ability with EC_{50} values ranging from 8.73 to 176.39 μM. Compounds **1–11** showed no cytotoxicity against cell lines A549, K562, ASPC, and H460 at 200 μM. Our current reports support the safety of *A. luchuensis* in food chemistry and confirm this fungus to be a new source of natural antioxidants.

1. Introduction

The fungi in the genus of *Aspergillus* have been reported to be a rich resource of biologically active secondary metabolites. Compounds with diverse chemical skeletons including alkaloids [1], terpenes [2], cerebroside analogues [3], polyketones [4], naphthoquinones [5] has been isolated from *Aspergillus* spp. *Aspergillus luchuensis* is an important and representative fungus in the black- and white-*koji* in China and Japan, and also often encountered in the meju and nuruk in Korea and Puerh tea in China. In the wine industry, *A. luchuensis* is used as an important additive in the Jiuqu due to its high production of enzymes for saccharification of raw materials (rice, barley, and sweet potatoes). Extractive analysis of strains of *A. luchuensis* confirmed its safety for food and beverage fermentations. There is no report of secondary metabolites from *A. luchuensis*. To clarify the secondary metabolites and their bioactivity, we conducted chemical investigation on a strain of *A. luchuensis* fermented on sterilized rice. HPLC and TLC analyses of the EtOAc extract indicated the presence of rich secondary metabolites. A detailed chemical investigation resulted in the isolation of four new diketopiperazine derivatives, one new methyl 4-(3-acetyl-2,6-dihydroxyphenyl)-2-methoxybutanoate, as well as six known compounds from the rice *koji* of this fungus. Herein we report the isolation, structure elucidation, anti-oxidative activity of these compounds.

2. Material and methods

2.1. General experimental procedures

Optical rotations were measured using a PerkineElmer 241 polarimeter. CD spectra were recorded on a JASCO J-815 spectropolarimeter. UV data and IR data were recorded on a Thermo Genesys-10S UV-vis spectrophotometer and a Nicolet IS5FTIR spectrophotometer, respectively. ^{1}H, ^{13}C NMR, DEPT spectra and 2D–NMR spectra were acquired with Bruker Avance-500 spectrometer. HR-TOF-MS data was recorded using an Agilent Accurate-Mass-Q-TOF LC/MS 6520 instrument. Preparative HPLC was performed on an Agilent 1200 HPLC system using a RP-18 column (250 × 10 mm, YMC Pack, 5 μm; detector: UV) with a flow rate of 2 mL/min.

2.2. Fungal material

The strain *A. luchuensis* used in this work was isolated from sake *koji*, and identified by one of co-authors (Junjie Han). The fungal strain was cultured on slants of potato dextrose agar at 25 °C for 10 d.

* Corresponding author at: State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, No. 1 Beichenxi Road, Chaoyang District, Beijing 100101, PR China.
E-mail address: liuhw@im.ac.cn (H. Liu).

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2.3. Fermentation

Rice (80 g) and distilled (100 mL) water were sterilized at 121 °C for 30 min. The cooked rice was cooled to room temperature and then inoculated with *A. luchuensis* spores to a final concentration of 1×10^5 spores/g substrate. The inoculated substrates were cultured at 25 °C for 30 days.

2.4. Extraction and isolation

The fermented rice (3.2 kg) was extracted repeatedly with ethyl acetate (3 × 3.0 L) at room temperature. The organic solvent was evaporated under vacuum to yield the crude extract (100.0 g). The crude extract was separated into 24 fractions on a silica gel column chromatography (1000 g, 100 * 8 cm) using gradient elution with petroleum ether-ethyl acetate (v/v, 50:1, 20:1, 10:1, 5:1; elution volume for each gradient was 5 L), and dichloromethane-methanol (v/v, 100:0, 80:1, 50:1, 30:1, 20:1, 15:1, 10:1, 8:1, 4:1, 0:1; elution volume for each gradient was 5 L). Fr.14 (6.3 g) was centrifuged to yield the precipitate (0.9 g) and supernatant (5.1 g). The precipitate was further purified by semi-preparative RP-HPLC (60% methanol in water) to give **10** (20.0 mg, t_R 14.5 min) and **11** (5.0 mg, t_R 15.6 min). The supernatant (5.1 g) was fractionated on a reversed-phase C18 silica column chromatography (80 g, 30 * 4 cm) eluted with methanol-H_2O in gradient eluent (v/v, 30:70, 40:60, 45:55, 50:50, 55:45, 70:30, 75:25, 80:20, 90:10, 100:0; elution volume for each gradient was 1 L) to giver 16 subfractions (Fr.14.1 to Fr.14.16). Fr.14.14 (151.0 mg) was then fractionated on a Sephadex LH-20 column (50 g 120 * 1.5 cm) eluted with dichloromethane/methanol (v/v, 1:1) to get 4 subfractions (Fr. 14.14.1 to Fr. 14.14.4). Fr.14.14.2 (95.2 mg) was finally purified by semi-preparative RP-HPLC (65% methanol in water) to give **3** (12.0 mg, t_R 30.8 min). Compound **6** (50.0 mg, t_R 25.4 min) was purified from Fr. 14.12 (130.6 mg) by semi-preparative RP-HPLC (59% methanol in water). Fr. 12 (14.0 g) was first fractionated on a reversed-phase C18 silica column chromatography (100 g 30 * 4 cm) eluted with methanol-H_2O in gradient eluent (v/v, 25:75, 35:65, 45:55, 48:52, 55:45, 65:35, 70:30, 78:22, 85:15, 90:10, 100:0; elution volume for each gradient was 2 L) to yield 15 subfractions (Fr. 12.1 to Fr. 12.15). Fr. 12.6 (50.0 mg), Fr. 12.7 (90.5 mg) and Fr. 12.8 (22.4 mg) were purified by semi-preparative RP-HPLC (30% acetonitrile in water, 34% acetonitrile in water and 32% methanol in water, respectively) to give **1** (5.4 mg, t_R 27.7 min), **4** (6.0 mg, t_R 28.1 min), and **2** (2.5 mg, t_R 29.1 min), respectively. Fr.10 (5.0 g) was fractionated on a reversed-phase C18 silica column chromatography (80 g, 30 * 4 cm) eluted with methanol-H_2O in gradient eluent (v/v, 20:80, 30:70, 40:60, 50:50, 55:45, 60:40, 70:30, 80:20, 100:0; elution volume for each gradient was 1 L) to afford 14 subfractions (Fr. 10.1 to Fr. 10.14). Compound **5** (5.0 mg, t_R 24.8 min) was obtained from Fr. 10.8 (50.8 mg) by semi-preparative RP-HPLC (30% acetonitrile in water). Fr. 10.4 (497.0 mg) was fractionated on a Sephadex LH-20 (100 g, 120 * 2 cm) column eluted with methanol to give **9** (277.0 mg, t_R 17.6 min). Fr. 10.6 (870.1 mg) was separated on a Sephadex LH-20 column (100 g, 120 * 2 cm) eluted with methanol to get 6 subfractions (Fr. 10.6.1 to Fr. 10.6.6). Fr. 10.6.2 (268.4 mg) was purified by semi-preparative RP-HPLC (46% methanol in water) to yield **7** (83.1 mg, t_R 21.4 min) and **8** (93.7 mg, t_R 20.7 min).

2.4.1. Luchunazine A (1)

Light yellow powder; $[\alpha]_D^{25}$ +8.0 (c 0.5, methanol); CD (c 0.83×10^{-3} M, methanol) λ_{max} ($\Delta\varepsilon$): 211 (+6.5), 236 (−14.0), 266 (+1.8), 325 (+0.9); UV (methanol) λ_{max} nm (log ε): 223 (4.2), 280 (3.5); IR (neat) ν_{max}: 3395, 2926, 1667, 1620, 1434, 1287, 1204, 1128, 1022, 745, 703 cm^{-1}; Positive HRTOFMS: *m/z* $[M + H]^+$ 600.2339 (calcd. for $C_{33}H_{34}N_3O_8$, 600.2340); ^1H and ^{13}C NMR spectroscopic data are listed in Table 1.

Table 1
^1H (500 MHz) and ^{13}C (125 MHz) NMR data for compounds **1** and **2** (DMSO-d_6).

Position	Compound **1**		Compound **2**	
	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)
1(N)				10.45 (s)
2	138.1		139.2	
3	105.7		104.3	
4	127.3		127.3	
5	118.2	7.26 (d, 8.0)	117.9	7.18 (o)
6	118.3	6.88 (t, 7.5)	118.3	6.88 (t, 7.4)
7	120.4	6.99 (t, 7.5)	120.3	6.98 (t, 7.4)
8	111.4	7.41 (d, 8.0)	111.2	7.36 (d, 8.0)
9	135.5		135.3	
10 (N)		6.82 (s)		6.53 (s)
11	54.5	3.48 (t, 6.9)	53.9	3.41 (t, 6.0)
12	27.9	3.10 (o)	28.1	2.85 (o)
				3.22 (dd, 14.5, 6.0)
13	168.2		168.3	
14 (N)		8.06 (s)		7.96 (s)
15	54.8	3.98 (t, 4.9)	54.6	3.82 (t, 5.0)
16	166.9		166.8	
17	37.4	2.83 (dd, 13.8, 4.9)	37.4	2.81 (o)
		3.02 (dd, 13.8, 4.9)		2.99 (dd, 13.8, 5.0)
18	136.2		136.1	
19	130.0	7.15 (o)	130.0	7.10 (o)
20	128.0	7.22 (o)	128.0	7.20 (o)
21	126.6	7.22 (o)	126.6	7.20 (o)
22	128.0	7.22 (o)	128.0	7.20 (o)
23	130.0	7.15 (o)	130.0	7.10 (o)
24	162.3		162.5	
25	115.5		113.8	
26	162.3		162.5	
27	108.1	6.47 (d, 8.8)	107.9	6.45 (d, 8.8)
28	131.5	7.65 (d, 8.8)	131.9	7.65 (d, 8.8)
29	112.6		112.6	
30	203.4		203.4	
31	26.2	2.50 (s)	26.1	2.50 (s)
32	27.3	5.03 (dd,10.1, 5.2)	27.2	5.01 (dd, 10.6, 5.5)
33	34.9	2.03 (ddd, 14.0, 10.3, 5.2)	33.8	2.27 (ddd, 13.9, 9.3, 5.5)
		2.94 (ddd, 14.0, 10.1, 4.2)		2.89 (o)
34	78.4	3.37 (o)	78.4	3.48 (dd, 9.3, 3.9)
35	173.7		172.6	
36	56.9	3.12 (s)	57.6	3.11 (s)
37			51.6	3.55 (s)

"o" signals overlapped.

2.4.2. Luchunazine B (2)

Light yellow powder; $[\alpha]_D^{25}$ +88.0 (c 0.5, methanol); CD (c 0.82×10^{-3} M, methanol) λ_{max} ($\Delta\varepsilon$): 215 (−7.6), 237 (+16.0), 271 (−1.0), 325 (−0.6); UV (methanol) λ_{max} nm (log ε): 223 (4.2), 280 (3.4); IR (neat) ν_{max}: 3272, 2926, 1671, 1619, 1437, 1291, 1204, 1132, 702 cm^{-1}; Positive HRTOFMS: *m/z* $[M + H]^+$ 614.2496 (calcd. for $C_{34}H_{36}N_3O_8$, 614.2497); ^1H and ^{13}C NMR spectroscopic data are listed in Table 1.

2.4.3. Luchunazine C (3)

White powder; $[\alpha]_D^{25}$ +198.0 (c 0.5, methanol); CD (c 0.53×10^{-3} M, methanol) λ_{max} ($\Delta\varepsilon$): 222 (+9.7), 244 (−47.4), 275 (+24.9), 301 (+18.1), 338 (−2.8); UV (methanol) λ_{max} nm (log ε): 204 (4.6), 295 (3.2); IR (neat) ν_{max}: 3353, 2930, 1731, 1672, 1614, 1434, 1317, 1207, 1127, 1089, 1024, 748, 703 cm^{-1}; Positive HRTOFMS: *m/z* $[M + H]^+$ 945.3818 (calcd. for $C_{54}H_{53}N_6O_{10}$, 945.3818); ^1H and ^{13}C NMR spectroscopic data are listed in Table 2.

2.4.4. Luchunazine D (4)

White powder; $[\alpha]_D^{25}$ −20.0 (c 0.5, DMSO); CD (c 0.75×10^{-3} M, methanol) λ_{max} ($\Delta\varepsilon$): 235 (+8.6), 283 (+1.3); UV (DMSO) λ_{max} nm (log ε): 210 (4.6), 312 (3.8); IR (neat) ν_{max}: 3195, 3057, 2967, 1667, 1454, 1336, 1320, 1241, 1206, 1092, 797, 745, 701 cm^{-1}; Positive HRTOFMS: *m/z* $[M + H]^+$ 665.2876 (calcd. for $C_{40}H_{37}N_6O_4$,