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New Antibacterial Dihydropyrones Induced by Co-Culture of *Penicillium crustosum* PRB-2 and *Penicillium citrinum* HDN11-186

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Abstract Two new dihydropyrones, rhytismatones C (**1**) and D (**2**), and a known compound, penicillenol A_1 (**3**), were isolated from the co-culture broth of the deep-sea-derived fungus *Penicillium crustosum* PRB-2 and *Suaeda salsa*-derived endophytic fungus *Penicillium citrinum* HDN11-186. Their structures were elucidated through comprehensive analysis of nuclear magnetic resonance (NMR) spectra and mass spectra. The absolute configurations of new compounds were determined by calculating the electronic circular dichroism (ECD) spectrum. UPLC-MS data showed that compounds **1**–**3** could only be detected in the media of co-culture, suggesting new biosynthetic pathways were activated in the co-cultured fungi. Compound **1** showed obvious antibacterial activities against *Proteus* sp. MMBC-1002 and *Bacillus subtilis* MMBC-1004 with minimum inhibitory concentration (MIC) both at 25 μmol L^{-1}.

Key words co-culture; *Penicillium crustosum*; *Penicillium citrinum*; dihydropyrones; antibacterial activity

1 Introduction

Fungi biosynthesize a variety of secondary metabolites that help them to survive in their habitats, and these metabolites have been proved to be an important source for the novel drug. Studies indicate that fungi possess abundant biosynthetic gene clusters. For example, at least 30 biosynthetic gene clusters have been detected in many *Aspergillus* spp. (Knowles *et al*., 2022). However, a great deal of gene clusters in fungi are silent under pure culture, largely because their strong biosynthetic potential was maintained through communication and interaction with neighbors in complex natural environment (Boddy, 2000; Qu *et al*., 2019). Thus, the co-culture strategy, which simulates the relationship of different fungi in their habitats, is an effective way to activate the silent biosynthetic pathways (Moody, 2014). Moreover, the natural products induced by co-culture strategy tend to exhibit excellent bioactivities, such as antimicrobial, anti-tumor and anti-fouling activities (Watanabe *et al*., 1982; Cho and Kim, 2012; Li *et al*., 2014; Knowles *et al*., 2019).

In our previous work, many novel bioactive compounds have been identified from *Penicillium crustosum* PRB-2, a deep-sea fungus which showed great biosynthesis potential (Wu *et al*., 2012, 2022; Yu *et al*., 2019, 2022). AntiSMASH analysis of the genome sequence of PRB-2 revealed the presence of at least 38 biosynthetic gene clusters, however, the metabolites encoded by most of these clusters were unknown (Fig.1). To tap the biosynthetic potential and search for more bioactive secondary metabolites, PRB-2 was further co-cultured with many other strains derived from diverse habitats. During this process, we found that co-culturing of PRB-2 and *Penicillium citrinum* HDN11-186, an endophytic fungus derived from *Suaeda salsa*, resulted in the production of new metabolites as indicated by the HPLC profiles. Further study of the co-cultivation system led to the discovery of two new dihydropyrones, rhytismatones C (**1**) and D (**2**), and a known compound penicillenol A_1 (**3**). Here, the details of isolation, structure elucidation, and bioactivity evaluation were reported.

2 Materials and Methods

2.1 General Experimental Procedures

UPLC-MS spectra were measured on Waters ACQUITY-SQ UPLC-mass spectrometer. HPLC was applied using an YMC ODS column (10 mm × 250 mm, 5 μm). NMR data

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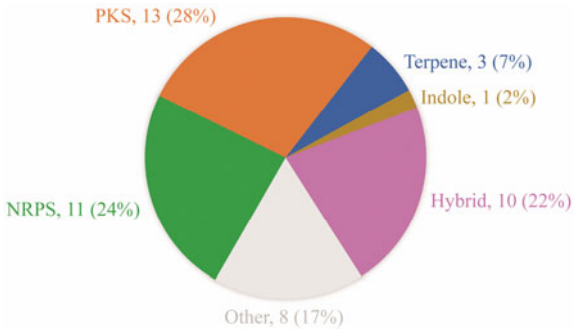

Fig.1 Types, quantity and corresponding proportion of gene clusters in PRB-2.

were measured on an Agilent DD2 spectrometer, using TMS as internal standard (500 MHz). ECD data were obtained on a JASCO J-715 spectrometer. UV spectra and optical rotations were recorded on Waters 2487 and JASCO P-1020, respectively. HRESIMS data were acquired by a Thermo Scientific LTQ-Orbitrap XL spectrometer and ESIMS data were tested on a Waters Micromass Q-TOF spectrometer.

2.2 Fungal Material

The strain HDN11-186 was isolated from the leave of *Suaeda salsa* collected from Dongying, Shandong, China, and was identified as *Penicillium citrinum* by analyzing ITS and *β*-tubulin gene sequences (accession number: OM757 912). The corresponding information of *P. crustosum* PRB-2 has been reported in our early research (Wu *et al*., 2012). Those fungal materials were deposited in our laboratory.

2.3 Fermentation and Extraction

PRB-2 and HDN11-186 were co-cultured in 500 mL flasks with 150 mL medium, consisting of glycerinum (20.0 g L^{-1}), peptone (2.0 g L^{-1}), yeast extract (2.0 g L^{-1}), and seawater. After the cultivation at 28℃, with shaking at 180 r min^{-1} for 9 days, the whole broth (20.0 L) was collected and filtered through cheesecloth. Then, the wet mycelia and the supernatant were extracted with MeOH and EtOAc, respectively, to obtain a crude extract, which was about 27 g (Yu *et al*., 2018).

2.4 Purification

The crude extract was fractionated into six fractions (fractions 1–6) by a C-18 ODS column using gradient elution with MeOH-H_2O (5%–100%) as the solvent. Based on the UPLC-MS data, fractions 2 (eluted with 20:80 MeOH-H_2O) and 5 (eluted with 80:20 MeOH-H_2O) were selected for further research. Fraction 2 was separated by HPLC (43:57 MeOH-H_2O, 3 mL min^{-1}) to furnish **1** (15.0 mg, t_R 21.0 min) and furnish **2** (30.0 mg, t_R 17.5 min). Fraction 5 was purified on HPLC (80:20 MeOH-H_2O, 3 mL min^{-1}) to furnish **3** (15.0 mg, t_R 20.0 min).

Rhytismatone C (1): pale yellow oil; $[\alpha]^{20}_D$ −46.8 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (logε): 235 (3.96), 268 (3.99) nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 269.1025 $[M-H]^-$ (calcd. for $C_{13}H_{17}O_6$, 269.1031).

Rhytismatone D (2): pale yellow oil; $[\alpha]^{20}_D$ −66.7 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (logε): 235 (3.95), 267 (3.99) nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 255.0878 $[M-H]^-$ (calcd. for $C_{12}H_{15}O_6$, 255.0874).

Table 1 ^1H (500 MHz) and ^{13}C (125 MHz) NMR data of compounds **1** and **2** (DMSO-d_6, δ ppm)

No.	1		2	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
2	171.6		172.4	
3	97.0		96.3	
4	192.6		193.3	
5	36.1	2.88 dd (3.3, 16.5) 2.59 dd (8.1, 16.5)	36.9	2.76 dd (2.8, 16.4) 2.37 dd (8.5, 16.4)
6	75.7	4.73 brs	75.9	4.60 brs
7	170.1		171.3	
8	51.7	3.61 s		
1'	193.2		193.4	
2'	37.4	2.71 t (7.4)	38.1	2.67 t (7.5)
3'	24.5	1.51 m	24.3	1.48 m
4'	31.1	1.27 overlap	31.1	1.26 overlap
5'	22.0	1.27 overlap	22.0	1.26 overlap
6'	13.8	0.85 t (6.8)	13.9	0.85 t (6.7)

2.5 ECD Calculations

Merck molecular force field was adopted for conformational search. The conformer with a distribution above 5.0% was re-optimized and calculated at B3LYP/6-31+G (d) level with PCM model for MeOH. ECD spectrum was obtained using SpecDis3 with the sigma/gamma setting as 0.4 eV and UV-shift setting as 5 nm for better comparison with the experimental data (Yu *et al*., 2016).

2.6 Assay of Cytotoxicity Inhibitory Activity and Antimicrobial Activities

The cytotoxicities were measured by methylthiazoletrazolium (MTT) or Sulforhodamine B (SRB) method, and the antimicrobial activities were tested in the 96 wells plate, as previously reported (Yu *et al*., 2018).

3 Results and Discussion

The two strains PRB-2 and HDN11-186 were co-cultured for 9 days at 28℃, with shaking at 180 r min^{-1}. Guided by UPLC-MS analysis, the total extract (27.0 g) was purified *via* column chromatography and semi-preparative HPLC to obtain the newly generated compounds **1**−**3** (Fig.2).

Rhytismatone C (**1**) was determined as $C_{13}H_{18}O_6$ based on the HREIMS signal at *m/z* 269.1025 $[M-H]^-$. The ^1H and ^{13}C NMR data (Table 1) suggested the existence of five nonprotonated sp^2 carbons (δ_C 193.2, 192.6, 171.6, 170.1, 97.0), one oxygenated methine (δ_C 75.7), five methylenes, and two methyls including one oxygenated (δ_C 51.7). The COSY correlation between H-2' and H-3', and the HMBC correlations from H_3-6' to C-4'/C-5', from H_2-3' to C-1'/C-4'/C-5', from H_2-2' to C-1'/C-4'/C-3 suggested the presence of an unsaturated aliphatic chain (C-3, C-1'–C-6') (Fig.3). The HMBC signals from H_3-8 to C-7 and from H_2-5 to C-4/C-6/C-7 suggested the presence of a fragment containing

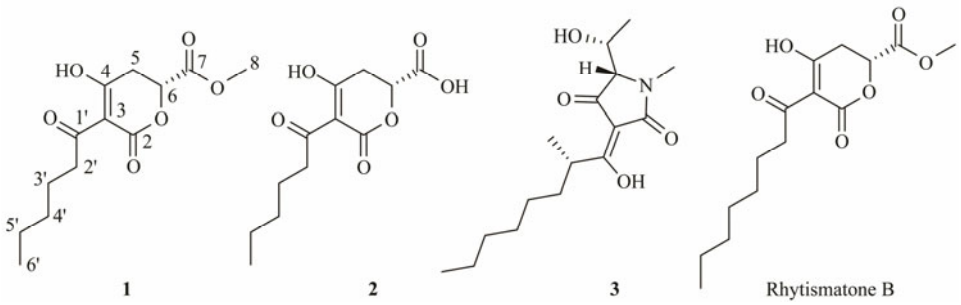

Fig.2 Structures of compounds **1**–**3** and similar compound rhytismatone B.

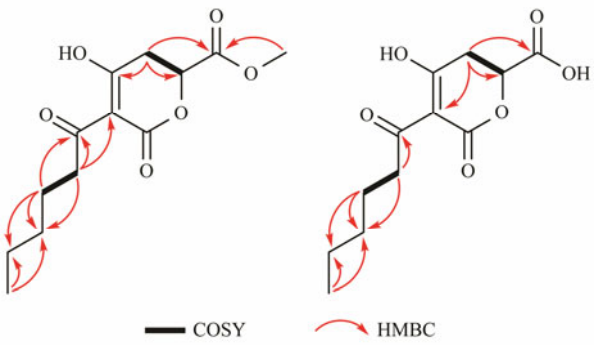

Fig.3 Key COSY and HMBC correlations of **1** and **2**.

methyl ester group (C-4–C-8) (Fig.3). Based on chemical shifts of C-6 (δ_C 75.7) and the left carbonyl (δ_C 171.6), we speculated that there was an ester fragment which link to C-6. At last, a trisubstituted-dihydropyrone-ring was deduced by comprehensive analysis of chemical shifts of C-2 (δ_C 171.6), C-3 (δ_C 97.0) and C-4 (δ_C 192.6), as well as the molecular weight (Fig.2; Table 1). These chemical shifts were consistent with that of rhytismatone B (C-2 (δ_C 174.1), C-3 (δ_C 99.6) and C-4 (δ_C 197.6) in CD_3OD), a compound with different length of unsaturated aliphatic chain (compound **1**: C_6; rhytismatone B: C_8) (Fig.2) (McMullin *et al*., 2017). The result of the chiral HPLC analysis indicated that compound **1** was a pure substance rather than an enantiomeric mixture, as evidenced by the presence of a major peak. Thus, the absolute stereochemistry of **1** was further determined by comparing the calculated ECD spectrum with the experimental one (Fig.4) (Yu *et al*., 2016). The ECD spectrum were calculated using the fragment **1a** as the truncated model structure of **1** (Fig.4). A conformer with a Boltzmann distribution 97.2% was obtained and calculated. As the calculated ECD spectrum of *R*-**1a** was identical to the experimental result of **1**, the absolute stereochemistry of **1** was determined as *R* (Fig.4).

Rhytismatone D (**2**) was determined as $C_{12}H_{16}O_6$ based on HREIMS signal at *m*/*z* 255.0878 $[M-H]^-$. The ^1H and ^{13}C NMR data (Table 1) of **2** suggested the existence of five nonprotonated sp^2 carbons (δ_C 193.4, 193.3, 172.4, 171.3, 96.3), one oxygenated methine (δ_C 75.9), five methylenes, one methyl, which were very similar to the result of compound **1**. Compared with **1**, the resonances at δ_H 3.61 (s) and δ_C 51.7 were disappeared in **2**, suggested that it was not esterified at C-7 (Fig.2). The analysis of COSY and HMBC spectra further confirmed our speculation (Fig.3). The presence of a main peak in the chiral HPLC analysis of compound **2** indicates that it is a pure substance instead of a mixture of enantiomers, too. Furthermore, the absolute stereochemistry of compound **2** was determined as *R*, based on the highly similarity of its ECD spectrum to that of compound **1**.

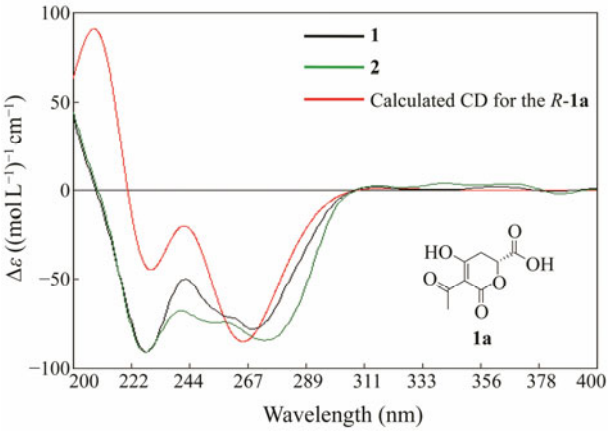

Fig.4 Experimental ECD spectra of compounds **1** and **2** and the calculated spectrum for *R*-**1a**.

Rhytismatones C (**1**) and D (**2**) were identified as new compounds with a dihydropyrone core structure. Natural products with the same core structure have been reported in other organisms and most of them showed remarkable antimicrobial activities. For example, alternaric acid from *Alternaria solani* showed inhibitory activities against *Absidia glauca* and *Myrothecium verrucaria*; rhytismatones A and B from *Rhytismataceae* sp. showed inhibitory activities against *Saccharomyces cerevisiae*; and podoblastins A−C from *Podophyllum peltatum* showed inhibitory activities against *Pyricularia oryzae*. Compound **3** was determined as penicillenol A_1 based on the coincident ^1H NMR data and the close optical rotation, which has been isolated from a *Penicillium* sp. and reported as a potent cytotoxic substance against HL-60 with the IC_{50} value of 0.76 μmol L^{-1} (Sengoku *et al*., 2010).

The cytotoxicities of **1** and **2** were measured using twelve cell lines (including HL-60, SH-SY5Y, Hela, L-02, *etc*.) by MTT or SRB method. However, both of them showed no activity at 30 μmol L^{-1}. Inspired by the antimicrobial activities of other dihydropyrone derivatives, the antimicrobial activities of **1** and **2** were measured using eight kinds of microorganisms including one kind of fungus, *Candida*

albicans MMBC-2001, and seven kinds of bacteria, including *Escherichia coli* MMBC-1001, *Proteus* sp. MMBC-1002, *B. subtilis* MMBC-1004, *Mycobacterium phlei* MMBC-1005, *Bacillus cereus* MMBC-1007, Methicillin-resistance coagulase negative *Staphylococci* (MRCNS) MMBC-1009, and *Acinetobacter baumannii* MMBC-1012. Compound **1** displayed obvious activities against *Proteus* sp. MMBC-1002 and *B. subtilis* MMBC-1004 with the MIC values both at 25.0 μmol L^{-1} (Table 2). Ciprofloxacin, the positive control, showed inhibitory effect on two bacteria with the MIC values of 0.2 μmol L^{-1} and 13.0 nmol L^{-1}.

The UPLC-MS profiles revealed that compounds **1**−**3** were not produced in mono-culture of these two fungi under the same condition used for co-culture. This indicated that the gene clusters synthesizing them were activated by the interaction of two fungi in co-culture condition (Fig.5).

Table 2 Antibacterial activities of compound **1**

Compound	MIC (μmol L^{-1})	
	Proteus sp. MMBC-1002	*B. subtilis* MMBC-1004
1	25.0	25.0
Ciprofloxacin	0.2	0.013

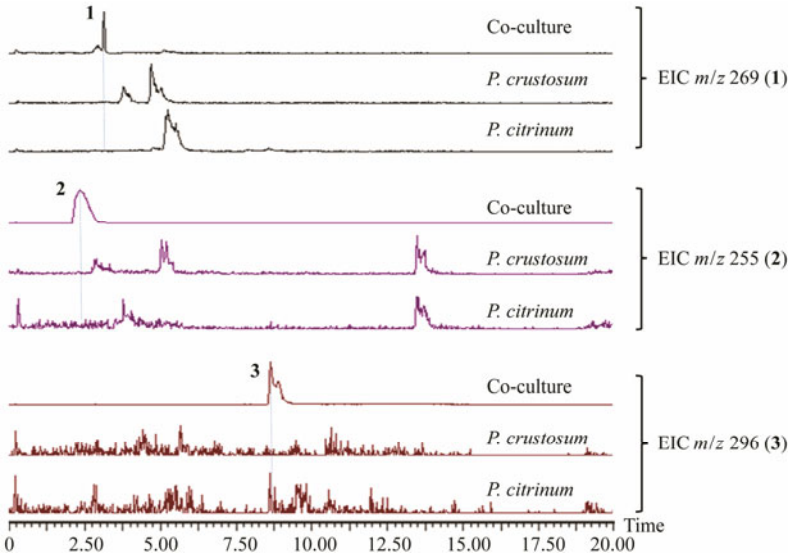

Fig.5 UPLC-MS analysis of the 9th day fermentation broth extracts from co-culture of *P. crustosum* PRB-2 and *P. citrinum* HDN11-186, as well as culturing them alone (concentration: 5 mg mL^{-1}, extracted negative ion peak ($[M-H]^-$) of compounds **1**−**3**).

4 Conclusions

Chemical investigation of the co-culture extract of the fungi PRB-2 and LD-11 led to the discovery of two new dihydropyrones, rhytismatones C (**1**) and D (**2**), as well as a known compound, penicillenol A_1 (**3**). The absolute configurations of **1** and **2** were elucidated by the calculation of ECD spectrum. Compound **1** showed obvious antibacterial effects on *Proteus* sp. MMBC-1002 and *B. subtilis* MMBC-1004 with the MIC values at 25 μmol L^{-1}. All three compounds were only produced in co-culture condition, suggested that co-culture strategy was an effective method to activate the silent biosynthetic pathways.

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