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Elucidation of Antifungal Metabolites Produced by *Pseudomonas aurantiaca* IB5-10 with Broad-Spectrum Antifungal Activity

Park, Gwee Kyo†, Jong-Hui Lim†, Sang-Dal Kim, and Sang Hee Shim*

School of Biotechnology, Yeungnam University, Gyeongsan, Gyeongbuk 712-729, Korea

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Antifungal metabolites were isolated from a culture of *Pseudomonas aurantiaca* IB5-10. Chemical structures of the metabolites were elucidated as phenazine-1-carboxylic acid (PCA; 1), 2-hydroxyphenazine (2-OH-PHZ; 2), and cyclo-(L-Pro-L-Val; 3), respectively, based on spectroscopic methods. Among them, 3 was isolated for the first time from this strain. The antifungal activities of 1–3 were evaluated against a variety of plant pathogens. To the best of our knowledge, the antifungal activities of 3 against plant fungal pathogens have been evaluated for the first time in this work. PCA (1) showed the most potent antifungal activities against *Phytophthora capsici*, *Rhizoctonia solani* AG-1(IA), and *Pythium ultimum* with MICs (μg/ml) of less than 1.0, 1.3, and 2.0, respectively. On the other hand, 2-OH-PHZ (2) showed potent antifungal activity against *R. solani* AG-1(IA) with the MIC (μg/ml) of 2.0, whereas it showed moderate antifungal activity against *P. ultimum* with the MIC (μg/ml) of 50.0. In addition, 3 showed antifungal activity against only *R. solani* AG-1(IA).

Keywords: *Pseudomonas aurantiaca*, antifungal activities, phenazine-1-carboxylic acid (PCA), 2-hydroxyphenazine (2-OH-PHZ), cyclo-(L-Pro-L-Val)

Some *Pseudomonas* spp., particularly fluorescent *Pseudomonas*, are known to be suitable to be used as agricultural biocontrol agents because they can produce a great deal of secondary metabolites to protect plants from pathogens [9]. Over 20 *Pseudomonas* species have been reported to produce more than 100 aromatic antibiotics [2]. The antibiotic compounds include phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), 2,4-diacetylphloroglucinol (Phl), pyocyanin, 2-acetamidophenol, pyrolnitrin, pyoluteorin, viscosinamide, tesin, *etc*. [3]. Among them, naturally occurring phenazines have gained increasing attention in recent years for their potential usage as antibiotics [10]. Over 50 different naturally occurring phenazines have been reported to be produced by *Pseudomonas* and *Streptomyces* spp. Naturally occurring phenazines have shown various biological activities such as antifungal, antibacterial, antitumor, antimalaria, antiparasitic, and enzyme-inhibiting activities [5]. In an effort to investigate the bioactive metabolites from phytopathogen-resistant microorganisms, a *Pseudomonas aurantiaca* strain was selected because it showed strong antifungal activity against a variety of plant fungal pathogens of agricultural importance. In this study, *P. aurantiaca* strain IB5-10 was chemically investigated and evaluated for its antibiotic activities. The antifungal metabolites were isolated using various steps of chromatographical methods and identified by spectroscopic methods. In addition, they were assayed for their antifungal activities against *Fusarium oxysporum*, *Phytophthora capsici*, *Corynespora cassiicola*, *Collectrichum acutatum*, *Botrytis cinerea*, *Rhizoctonia solani* AG-1(IA), *Rhizoctonia solani* AG-1(IB), and *Pythium ultimum*.

P. aurantiaca IB5-10 (KACC No. 91561P) was isolated for the plant growth-promoting rhizobacterium (PGPR) from a coastal sand dune in the East Coast of Korea [7]. This PGPR strain was cultured in Luria–Bertani (LB) broth medium.

The cultured *P. aurantiaca* IB5-10 was extracted with ethyl acetate (EtOAc) to yield an EtOAc layer (250 mg). The EtOAc-soluble fraction was subjected to silica gel (70–230 mesh; Merck) column chromatography with elution of a gradient of $CHCl_3$-MeOH to give 8 fractions (Fr. 1~Fr. 8). Fr. 2 was further subjected to semipreparative reverse-phase HPLC (Luna 5u Phenyl-Hexyl column; 250 × 10.00 mm; flow rate, 2 ml/min; 35–60% ACN in H_2O for 25 min; UV detection at 254 nm) to afford phenazine-1-carboxylic acid (PCA) (**1**) (1.5 mg, t_R = 22.5 min). Fr. 5

*Corresponding author
Phone: +82-53-810-3028; Fax: +82-53-810-4769;
E-mail: shshim29@ynu.ac.kr

†Both authors contributed equally to this work.

was also subjected to semipreparative reverse-phase HPLC (Luna 5u Phenyl-Hexyl column; 250 × 10.00 mm; flow rate, 2 ml/min; 30–55% ACN in H_2O for 25 min; UV detection at 254 nm) to afford 2-hydroxyphenazine (2-OH-PHZ) (**2**) (2.0 mg, t_R = 16.5 min). Fr. 6 was subjected to semipreparative reverse-phase HPLC (Luna 5u Phenyl-Hexyl column; 250 × 10.00 mm; flow rate, 2 ml/min; 5–30% ACN in H_2O for 20 min, followed by 30–50% ACN in H_2O for 20 min; UV detection at 210 nm) to afford cyclo-(L-Pro-L-Val) (**3**) (2.0 mg, t_R = 19.5 min). The chemical structures of the antifungal metabolites were determined by spectroscopic analyses methods such as ^{1}H-NMR, ^{13}C-NMR, and UV.

Phenazine-1-carboxylic acid (PCA) (1): Yellow amorphous powder; ^{1}H-NMR ($CDCl_3$, 600 MHz) δ 8.98 (1H, dd, *J* = 7.2, 1.2 Hz, H-4), 8.53 (1H, dd, *J* = 9.0, 1.2 Hz, H-2), 8.35 (1H, d, *J* = 8.4 Hz, H-6), 8.29 (1H, d, *J* = 8.4 Hz, H-9), 8.01 (3H, m, H-3, 7, 8); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ 165.9 (C-11), 144.1 (C-10a), 143.4 (C-9a), 140.1 (C-5a), 139.8 (C-4a), 137.4 (C-3), 135.1 (C-4), 133.2 (C-9), 131.7 (C-6), 130.2 (C-8), 130.1 (C-2), 128.0 (C-7), 124.9 (C-1); UV (MeOH) λ_{max} (log ε) 210 (4.30), 249 (4,77), 365 (4.06)

2-Hydroxyphenazine (2-OH-PHZ) (2): Yellow crystals; ^{1}H-NMR (CD_3OD, 600 MHz) δ 8.16 (1H, d, *J* = 9.0 Hz, H-6), 8.11 (1H, d, *J* = 7.8 Hz, H-9), 8.09 (1H, d, *J* = 9.6 Hz, H-4), 7.87 (1H, t, *J* = 7.8 Hz, H-8), 7.81 (1H, t, *J* = 8.4 Hz, H-7), 7.57 (1Hs, dd, *J* = 9.6, 3.0 Hz, H-3), 7.35 (1H, d, *J* = 3.0 Hz, H-1); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ 158.1 (C-2), 146.3 (C-10a), 144.1 (C-5a), 142.5 (C-4a), 141.4 (C-9a), 132.2 (C-4), 131.6 (C-8), 130.3 (C-6), 130.2 (C-9), 129.0 (C-7), 128.0 (C-3), 107.5 (C-1); UV (MeOH) λ_{max} (log ε) 210 (3.65), 251 (3.77), 358 (3.18)

Cyclo-(L-Pro-L-Val) (3): colorless crystals; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 5.66 (1H, br s, NH), 4.06 (1H, t, *J* = 7.2 Hz, H-6), 3.92 (1H, s, H-3), 3.56 (2H, m, H_2-9), 2.62 (1H, m, H-1'), 2.36 (1H, m, H-7), 1.84–2.02 (3H, m, H-7, H_2-8), 1.04 (3H, d, *J* = 7.2 Hz, H_3-2'), 0.88 (3H, d, *J* = 6.9 Hz, H_3-3'); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ 169.8 (C-2), 164.8 (C-5), 60.3 (C-3), 58.8 (C-6), 45.1 (C-9), 28.5 (C-7), 28.3 (C-1'), 22.3 (C-8), 19.3 (C-2'), 16.0 (C-3'); $[\alpha]_D^{21}$ -57.04° (C 1.0 × 10^{-3}, $CHCl_3$), -160.34° (C 1.0 × 10^{-3}, MeOH).

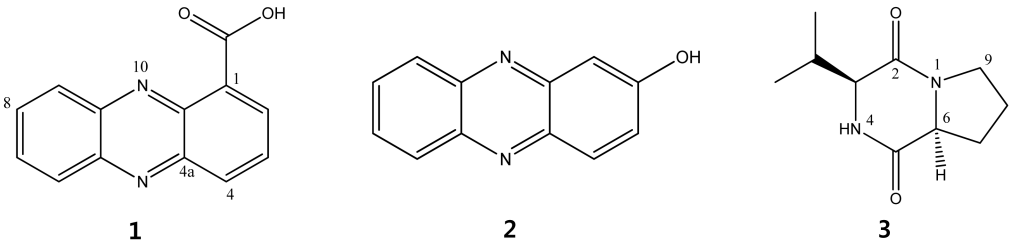
Compound **1** was obtained as a yellow amorphous powder. The structure of **1** was elucidated by ^{1}H-NMR and ^{13}C-NMR spectroscopies. The ^{1}H-NMR spectrum showed signals for a 1,2-disubstituted benzene moiety [at δ 8.35 (1H, d, *J* = 8.4 Hz, H-6); 8.29 (1H, d, *J* = 8.4 Hz, H-9); 8.01 (2H, m, H-7, 8)] and a 1,2,3-trisubstituted benzene moiety [at 8.98 (1H, dd, *J* = 7.2, 1.2 Hz, H-4); 8.53 (1H, dd, *J* = 9.0, 1.2 Hz, H-2); 8.01 (1H, m, H-3)]. The resonances at δ 8.89~8.3 implied that **1** is a nitrogen-containing heteroaromatic compound. On the basis of ^{1}H spectral resonances, **1** was presumed to be a derivative of phenazine, which was supported by a strong absorption peak at 249 nm accompanied by a broad peak at 365 nm in UV spectral analysis. The ^{13}C NMR spectrum showed the presence of 13 carbon resonances in **1**. Among them, four carbon signals at 144.1, 143.4, 140.1, and 139.8 indicated that they are attached to a nitrogen atom. The sp^2 quaternary carbon at 124.9 was presumed to be attached to the carboxylic acid. On the basis of the spectral data together with the literature values, **1** was identified as phenazine 1-carboxylic acid [5].

Compound **2** was obtained as a yellow crystal. The ^{1}H-NMR spectrum of **2** was similar to that of **1**, an indication that **2** is also a derivative of phenazine. The difference between **1** and **2** in ^{1}H-NMR was that signals for a 1,2,5-trisubstituted benzene moiety [at 8.09 (1H, d, *J* = 9.6 Hz, H-4); 7.57 (1H, dd, *J* = 9.6, 3.0 Hz, H-3); 7.35 (1H, d, *J* = 3.0 Hz, H-1)] appeared instead of those for the 1,2,3-trisubstituted benzene moiety in **1**. The *meta*-coupled methine proton signal at δ 7.35 suggested the attachment of a functional group to C-2. In the ^{13}C-NMR spectrum of **2**, twelve sp^2 carbon signals appeared. Among them, a highly upfield-shifted signal at δ 107.5 suggested that an electron-releasing group was attached to C-2. A hydroxy group was presumed to be positioned at C-2, which was supported by a carbon resonance at δ 158.1. Therefore, **2** was identified as 2-hydroxyphenazine and its spectral data were in good agreement with those of the literature [1, 6].

Compound **3** was obtained as a white amorphous powder. In the ^{1}H-NMR spectrum, two characteristic α proton signals in amino acids appeared at δ_H 4.06 and 3.92, respectively. In addition, two amide carbon signals appeared at δ_C 169.8 and 164.8, respectively, indicating that **3** is a dipeptide. Two doublet methyl groups at δ_H 1.04 and 0.88 and a methine multiplet signal at δ_H 2.62 indicating the presence of an isopropyl group suggested that one amino acid residue in **3** is a valine. In addition, signals for three sp^3 vicinally coupled methylene group suggested that the other amino acid residue is a proline. The absolute configuration of this peptide was elucidated by measuring its optical rotation. By comparison with the literature data, **3** was identified as a known cyclic dipeptide, cyclo-(L-Pro-L-Val) (Fig. 1) [7, 14].

PCA (**1**) and 2-OH-PHZ (**2**) have been reported to be isolated from the extracts of *P. aurantiaca* [12]. However, to the best of our knowledge, cyclo-(L-Pro-L-Val) (**3**) was isolated from *Pseudomonas* spp. for the first time.

The isolated compounds **1–3** were evaluated for antifungal activities against a variety of plant pathogenic fungal strains including *Fusarium oxysporum* f. sp. *lycoperisici* (KACC No. 40032), *Phytophthora capsici* (KACC No. 40473), *Corynespora cassiicola* (KACC No. 40964), *Collectrichum acutatum* (KACC No. 40042), *Botrytis cinerea* (KACC No. 40574), *Rhizoctonia solani* AG-1(IA) (KACC No. 40101), *Rhizoctonia solani* AG-1(IB) (KACC No. 40111), and *Pythium ultimum* (KACC No. 40705). The plant pathogenic fungal strains were obtained from the RDA-Genebank



1 **2** **3**

Fig. 1. Chemical structures of the isolated metabolites, phenazine-1-carboxylic acid (PCA; **1**), 2-hydroxyphenazine (2-OH-PHZ; **2**), and cyclo-(L-Pro-L-Val) (**3**).

Information Center (Rural Development Administration, Suwon, Korea) and routinely cultured in potato dextrose agar (PDA) medium for measuring antifungal activities and minimal inhibitory concentrations (MICs). The antifungal activities were determined by disc-paper method.

The purified antifungal compounds, which were dissolved in DMSO to yield 100 µl (1/10 dilution µg/ml), were loaded on sterile paper discs (8 mm in diameter). The controls were obtained by applying 100 µl of DMSO. The treated paper discs were placed in the 3 cm separated from a growing edge of the mycelia colony. Antifungal activity was observed by formation of a zone of inhibition of mycelia growth. To determine the minimum inhibitory concentration (MIC) of the antifungal compounds, the purified metabolites dissolved in DMSO were serially diluted to give the concentrations of 1/10, 25, 50, 100, 250, 500, 750, and 1,000 (dilution µg/ml) and loaded on paper discs. The discs were placed on PDA inoculated with zoospores of each pathogen and incubated at 28°C for 3 days. The MICs were estimated in µg/ml, when no visible germination of zoospores was observed.

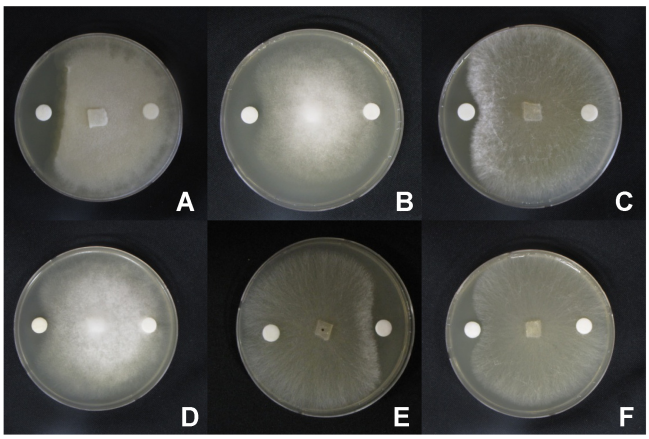


Fig. 2. Antifungal activities of the purified metabolites (**1–3**). (**A**), Inhibition of mycelia grown of *P. capsici* on PDA by metabolite **1**; (**B**), Inhibition of *P. ultimum* by **1**; (**C**), Inhibition of *R. solani* by **1**; (**D**), Inhibition of *P. ultimum* by **2**; (**E**), Inhibition of *R. solani* by **2**; (**F**), Inhibition of *R. solani* by **3**. Concentrations of all the applied samples, 0.01 µg/µl.

The antifungal activities of **1–3** are shown in Fig. 2. Compounds **1–3** showed antifungal activities against *P. capsici*, *R. solani* AG-1(IA), and *P. ultimum* among the tested plant pathogens, and their MICs were evaluated. PCA (**1**) was strongly effective against *P. capsici, R. solani* AG-1(IA), and *P. ultimum* with the MIC values (µg/ml) of less than 1.0, 1.3, and 2.0, respectively, but it was not effective against the other tested plant fungal pathogens. Compound 2-OH-PHZ (**2**) was effective against *R. solani* AG-1(IA) and *P. ultimum* with the MIC values of 2.0 and 50.0, respectively. Compound **3** was effective against only *R. solani* AG-1(IA) with the MIC of 4.0 µg/ml (Table 1 and Fig. 2).

Even though the antifungal activities of PCA (**1**) against a variety of plant fungal pathogens have been reported [5], little has been known about the *in vitro* antifungal activity against *C. cassiicola, C. acutatum, B. cinerea, R. solani* AG-1(IA), and *R. solani* AG-1(IB). There are reports on the antifungal activities of PCA against *F. oxysporum, P. capsici,* and *P. ultimum* [5]. The results agreed with ours. In addition, there are some reports on broad-spectrum *in vitro* antifungal activities of 2-OH-PHZ (**2**) [8, 12]. However, to our knowledge, this is the first report on *in vitro* antifungal activities of **2** against *C. cassiicola, C. acutatum, B. cinerea, R. solani* AG-1(IA), and *R. solani* AG-1(IB).

Cyclo-(L-Pro-L-Val) (**3**), which was first isolated from *Streptomyces* sp. S-580 [13], has never been reported to have antifungal activity. To our knowledge, this is the first report on the evaluation of *in vitro* antifungal activities of cyclo-(L-Pro-L-Val) against *F. oxysporum, P. capsici, C. cassiicola, C. acutatum, B. cinerea, R. solani* AG-1(IA),

Table 1. Minimum inhibitory concentrations (MICs) of the isolated metabolites.

	Minimum inhibitory concentration (µg/ml)		
	1	2	3
P. capsici	<1.0	-	-
R. solani AG-1(IA)	1.3	2.0	4.0
P. ultimum	2.0	50.0	-

-, No inhibition.

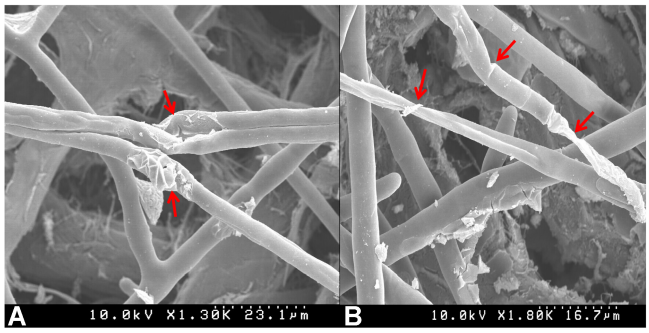


Fig. 3. Morphology of hyphae of *R. solani* AG-1(IA) treated with cyclo-(L-Pro-L-Val)(**3**).

R. solani AG-1(IB), and *P. ultimum*. Compound **3** showed inhibitory activity against only *R. solani* AG-1(IA). Microscopic observation was conducted to study the antifungal mechanism of **3**. For scanning electron microscopy (SEM), a culture of *R. solani* AG-1(IA) grown on PDA medium plates was sprayed with 100 μl of compound **3** solution and incubated for 24 h. A section of 1 cm^2 was transversely segmented and fixed in 4% glutarylaldehyde for 3 h. After washing with distilled water, the specimen was dehydrated in a graded ethanol series up to 100%, critical-point dried, and platinum-coated using an ion sputter coater. The specimen was observed under a Hitachi S-4100 FE-SEM/EDS. As shown in Fig. 3, the cell wall was collapsed, broken down (A), and the hyphae shrunken and tubular-deflated (B). Thus, **3** might possibly exhibit antifungal activity through inhibition of cell wall synthesis, like many other antifungal agents. Interestingly, **3** showed selective inhibitory activity to *R. solani* AG-1(IA) rather than *R. solani* AG-1(IB). *R. solani* is divided into 12 anastomosis groups (AG1-11 and BI) and AG-1 can be subdivided into three subgroups (IA, IB, and IC) depending on their pathogenicity [11]. Even though AG-1(IA) and (IB) belong to the same species, symptoms caused from both fungi have been known to be different. AG-1(IA) is referred to as a sheath-blight fungus, whereas AG-1(IB) is referred to as a web-blight fungus according to the symptoms they result in. It was reported that AG-l(IA) has physiologically and ecologically distinctive characteristics and specificity as pathogens, compared with IB and IC [11]. Many genetic studies on AGs and their intraspecific groups IA~IC have been carried out [4]. Homology of the DNA sequence was studied to determine the genetic relationships among AG-1(IA) and (IB) [4]. Comparatively low values of homology were observed between IA and IB [4]. Thus, there might be differences between IA and IB in cell wall components or in genes of enzymes associated with cell wall synthesis, which cause different resistance to cyclo-(L-Pro-L-Val) (**3**).

In conclusion, PCA (**1**), 2-OH-PHZ (**2**), and cyclo-(L-Pro-L-Val) (**3**) produced by *P. aurantiaca* IB5-10 can be used as effective biological control candidates against devastating plant fungal pathogens.

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