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BRIEF COMMUNICATION

Two new phenazine metabolites with antimicrobial activities from soil-derived *Streptomyces* species

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Abstract

Two new phenazine metabolites, 6-hydroxyphenazine-1-carboxamide (**1**) and methyl 6-carbamoylphenazine-1-carboxylate (**2**), were isolated from a soil-derived *Streptomyces diastaticus* subsp. *ardesiacus* strain YIM PH20246, and their structures were elucidated by extensive spectroscopic data analysis. The antimicrobial activities of the isolates were assayed. Compound **1** showed moderate antifungal and antibacterial activities against *Fusarium oxysporum* (ATCC 7808), *Fusarium solani* (ATCC 36031) and *Plectosphaerella cucumerina* (local isolate), *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus albus* (ATCC 10231), respectively. Compound **2** exhibited moderate antifungal and antibacterial activities against *F. oxysporum, F. solani*, and *Phoma herbarum, S. aureus, S. albus*, and *Bacillus subtilis*, respectively.

The actinomycetes, particularly species from the genus *Streptomyces*, have proved to be a tremendous high-impact source of valuable chemicals. They have yielded many clinically essential antimicrobial compounds, including streptomycin, actinomycin, and streptothricin. [1, 2] As part of our continuous search for further bioactive secondary metabolites from the genus Streptomyces, [3] two new phenazine alkaloids, 6-hydroxyphenazine-1-carboxamide (**1**) and methyl 6-carbamoylphenazine-1-carboxylate (**2**) (Fig. 1) were obtained from the fermentation broth of *Streptomyces diastaticus* subsp. *ardesiacus* strain YIM PH20246. In this paper, we report the fermentation, isolation, structural elucidation, and antifungal and antibacterial activities of compounds **1** and **2**.

The producing organism was isolated from the rhizosphere soil sample of *Panax notoginseng* collected in Wenshan, Yunnan Province, China. The strain was identified as *S. diastaticus* subsp. *ardesiacus* according to morphological characteristic and analysis of its 16S rRNA gene sequence (accession number MH600064). Phylogenetic analysis showed that the strain shared 98.88% 16S ribosomal RNA gene sequence similarity with the closely related strains *S. diastaticus* subsp. *ardesiacus* NRRL B-1773^T (accession number DQ026631). Additionally, the strain was examined for a number of key phenotypic properties known to be of value in streptomycete systematics, and the presence of L,L-diaminopimelic acid in the peptidoglycan together with its colonial characteristics supported its assignment to the genus *Streptomyces*.

A slant culture of the strain was inoculated into 500 ml-Erlenmeyer flasks (70×) containing 100 ml of seed medium composed of yeast extract 0.4%, glucose 0.4%, malt extract 0.5%, multiple vitamins solution 0.35 ml l^{-1} (consisting of 1.0 mg l^{-1} vitamin B_1, 1.0 mg l^{-1} vitamin B_2, 1.0 mg l^{-1} vitamin B_3, 1.0 mg l^{-1} vitamin B_6, 1.0 mg l^{-1} phenylalanine, 0.3 mg l^{-1} alanine, and 1.0 mg l^{-1} biotin), pH 7.2 with no adjustment and cultured for 2 days at 28 °C on a rotary shaker at 220 r.p.m. This seed culture was used to inoculate the fermentation medium with 10% volume. The fermentation was carried out in 1000 ml-Erlenmeyer flask (350×)

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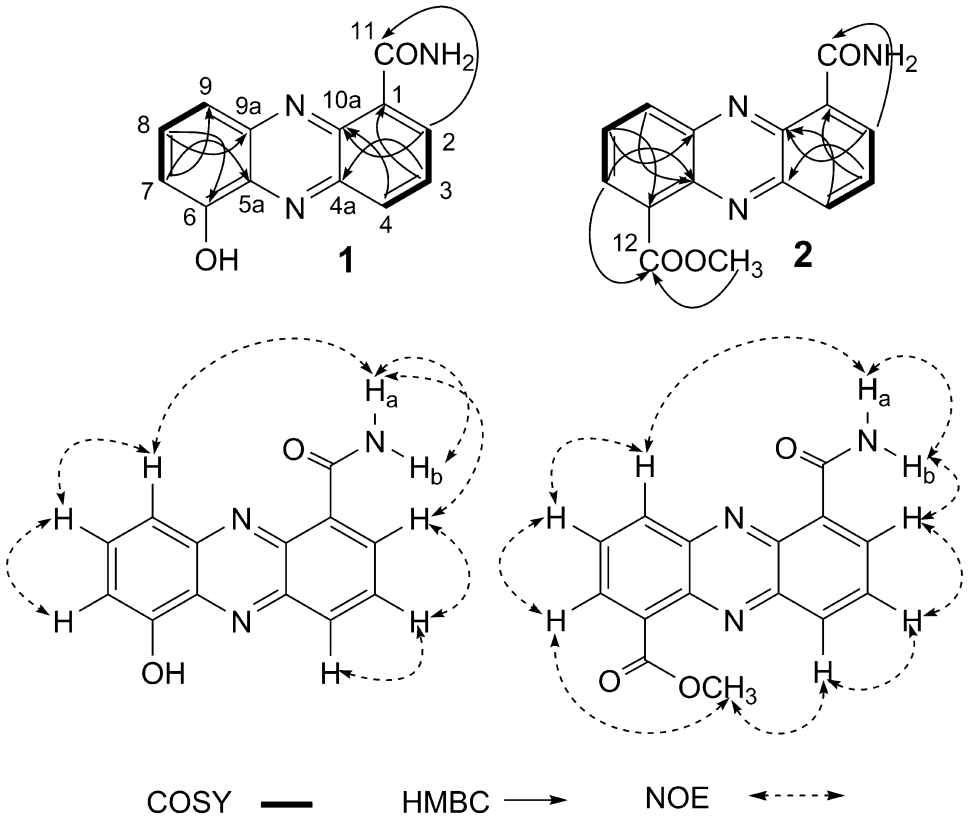

Fig. 1 Main COSY, HMBC, and NOE correlations of compounds **1** and **2**

containing 200 ml of fermentation medium containing starch 2.4%, beef extract 0.3%, glucose 0.1%, yeast extract 0.5%, peptone 0.3%, $CaCO_3$ 0.4%, pH 7.0 with no adjustment and cultured for 6 days at 28 °C on a rotary shaker at 220 r.p.m. for upscale fermentation.

The completed fermentation broth (70 l) was separated into filtrate and mycelium by centrifugation. After removal of the mycelium, the culture filtrate was extracted with ethyl acetate; the ethyl acetate-soluble portion was concentrated under reduced pressure to yield 14.6 g crude extract. The dried crude extract was then separated by silica gel column chromatography ($CHCl_3$-MeOH, gradient 80:1-10:1 (v/v)) into five fractions. The further isolation was based on the TLC detection. Targeting several colored compounds, fraction 3 was subjected to gel chromatography on Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) (MeOH) to produce two fractions (Fr. 3.1 and Fr. 3.2), Fr. 3.2 was then purified by silica gel column chromatography (petroleum ether-ethyl acetate 8:1 (v/v)) and Sephadex LH-20 chromatography to obtain 6 mg of compound **1**. Fraction 5 was applied to gel chromatography on Sephadex LH-20 (MeOH) to produce three fractions (Fr. 5.1 to Fr. 5.3), fraction 5.3 was further purified by silica gel column chromatography (petroleum ether-ethyl acetate 10:1 (v/v)) delivered 8 mg of compound **2**.

Compound **1** was obtained as an orange red amorphous powder. UV (MeOH) λ_{max} (log ε) 207 (4.59), 249 (4.34), 266 (4.59), 368 (3.86), 436 (3.36) nm. IR (KBr)ν_{max} 3442, 1661, 1625, 1525, 1474 cm^{-1}. High resolution electrospray mass spectroscopy (ESI HRMS) *m/z* 240.0768 $[M + H]^+$ (calcd for $C_{13}H_{10}N_3O_2$, 240.0768). For ^1H and ^{13}C NMR data see Table 1.

The IR spectrum of compound **1** indicated the presence of a hydroxyl group (3442 cm^{-1}), a carboxamide group (1661 cm^{-1}) and aromatic rings (1625, 1525, 1474 cm^{-1}). The UV spectrum of compound **1** showed peaks typical for phenazines at 266, 368, and 436 nm. [4] The ^1H NMR (in MeOH-d_4) spectrum of compound **1** (Table 1) showed six aromatic proton signals at δ 8.85 (1 H, dd, $J = 7.3$, 1.3 Hz), 8.53 (1 H, brd, $J = 8.6$ Hz), 8.01 (1 H, dd, $J = 8.6$, 7.3 Hz), 7.88 (1 H, dd, $J = 8.6$, 7.6 Hz), 7.81 (1 H, brd, $J = 8.6$ Hz), 7.26 (1 H, brd, $J = 7.6$ Hz). The ^{13}C NMR (in MeOH-d_4) data suggested thirteen carbons altogether, and the DEPT and HSQC experiments showed six aromatic methines. Furthermore, seven sp^2 nonprotonated carbons were presented, including one carboxamide carbon at δ 169.2 and six aromatic quaternary carbons at δ 154.8, 144.0, 143.0, 142.4, 137.1, and 130.4, respectively. The ^1H and ^{13}C NMR data suggested that compound **1** possesses phenazine skeletons like the related compound phencomycin. [5] The ^1H–^1H COSY (in MeOH-d_4) correlations were observed from δ_H 8.01 to 8.85 and 8.53, and from δ_H 7.88 to 7.81, and 7.26 suggested exsiting two ABC system aromatic rings in compound **1**. The two substituents were speculated as

Table 1 ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data for compounds **1** and **2**

position	1[a]		1[b]		2[b]	
	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	130.4, C		130.6, C		131.3, C	
2	136.7, CH	8.85, dd (7.3, 1.3)	134.3, CH	8.69, dd (7.3, 1.3)	134.7, CH	8.70, dd (6.9, 1.7)
3	130.7, CH	8.01, dd (8.6, 7.3)	129.6, CH	8.04, dd (8.6, 7.3)	131.1, CH	8.10, dd (8.6, 6.9)
4	135.6, CH	8.53, brd (8.6)	133.3, CH	8.46, dd (8.6, 1.3)	133.2, CH	8.40, dd (8.6, 1.7)
4a	143.0, C		141.2, C		142.7, C	
5a	137.1, C		135.7, C		139.7, C	
6	154.8, C		153.9, C		131.7, C	
7	111.9, CH	7.26, brd (7.6)	111.0, CH	7.25, brd (7.6)	132.1, CH	8.30, dd (6.9, 1.3)
8	134.7, CH	7.88, dd (8.6, 7.6)	133.3, CH	7.87, dd (8.6, 7.6)	130.9, CH	8.08, dd (8.6, 6.9)
9	120.4, CH	7.81, brd (8.6)	118.9, CH	7.80, brd (8.6)	132.7, CH	8.60, dd (8.6, 1.3)
9a	144.0, C		142.3, C		140.8, C	
10a	142.4, C		140.3, C		140.4, C	
11	169.2, C		165.9, C		165.6, C	
12					166.6, C	
OCH_3					52.7, CH_3	4.01, s
$CONH_a$				9.83, brs		9.61, brs
$CONH_b$				8.08, brs		8.14, brs
OH				10.81, brs		

[a]in MeOH-d_4

[b]in DMSO-d_6

carboxamide and hydroxyl groups according to the molecular formula. As shown in Fig. 1, HMBC (in MeOH-d_4) correlations between H-2 (δ 8.85) with C-11 (δ 169.2), C-10a (δ 142.4), C-4 (δ 135.6); H-3 (δ 8.01) with C-1 (δ 130.4), C-4a (δ 143.0); H-4 (δ 8.53) with C-2 (δ 136.7); H-9 (δ 7.81) with C-7 (δ 111.9), H-8 (δ 7.88) with C-6 (δ 154.8), C-9a (δ 144.0); H-7 (δ 7.26) with C-9 (δ 120.4), C-5a (δ 137.1) assigned the ^{1}H and ^{13}C NMR data of two aromatic rings. Key NOE correlations in DMSO-d_6 (Fig. 1) observed from CO–NH_a (δ 9.83) to H-2 (δ 8.69), H-9 (δ 7.80), and CO–NH_b (δ 8.08) illustrated that the carboxamide and hydroxyl were located at C-1 and C-6, respectively. Therefore, the structure of compound **1** was determined to be 6-hydroxyphenazine-1-carboxamide (Fig. 1) by extensive 1D and 2D NMR data together with IR absorption and UV maximum peak.

Compound **2** was obtained as a yellow amorphous powder. UV (DMSO) λ_{max} (log ε) 257 (4.43), 369 (4.13), 393 (3.66) nm. IR (KBr)ν_{max} 1732, 1674, 1617, 1533, 1450 cm^{-1}. ESI HRMS *m/z* 282.0873 $[M + H]^+$ (calcd for $C_{15}H_{12}N_3O_3$, 282.0873). For ^{1}H and ^{13}C NMR data see Table 1.

The molecular formula of compound **2** was established as $C_{15}H_{11}N_3O_3$ by ESI HRMS. The IR spectrum of compound **2** indicated the presence of a carbomethoxy group (1732 cm^{-1}), a carboxamide group (1674 cm^{-1}) and aromatic rings (1617, 1533, 1450 cm^{-1}). The UV spectrum of compound **2** is very similar to that of compound **1**, indicating a further phenazine derivative. The ^{1}H NMR spectrum of compound **2** (Table 1) showed six aromatic proton signals at δ 8.70 (1 H, dd, $J = 6.9$, 1.7 Hz), 8.60 (1 H, dd, $J = 8.6$, 1.3 Hz), 8.40 (1 H, dd, $J = 8.6$, 1.7 Hz), 8.30 (1 H, dd, $J = 6.9$, 1.3 Hz), 8.10 (1 H, dd, $J = 8.6$, 6.9 Hz), 8.08 (1 H, dd, $J = 8.6$, 6.9 Hz) and one methoxy signals at δ 4.01 (3 H, s). The ^{13}C NMR data suggested fifteen carbons altogether, and the DEPT and HSQC experiments showed six aromatic methines at δ 134.7, 133.2, 132.7, 132.1, 131.1, and 130.9, respectively. Furthermore, eight sp^2 non-protonated carbons were presented, including one carbomethoxy carbon at δ 166.6, one carboxamide carbon at δ 165.6 and six aromatic quaternary carbons at δ 142.7, 140.8, 140.4, 139.7, 131.7, and 131.3, respectively. ^{1}H and ^{13}C NMR spectrum data of compound **2** were very similar to those of compound **1** (Table 1) suggest that compound **2** also possesses phenazine skeleton like compound **1**. As shown in Fig. 1, ^{1}H–^{1}H COSY correlations were observed from δ_H 8.10 to 8.70 and 8.40, from δ_H 8.08 to 8.60 and 8.30, and HMBC correlations between H-2 (δ 8.70) with C-11 (δ 165.6), C-10a (δ 140.4), C-4 (δ 133.2); H-3 (δ 8.10) with C-1 (δ 131.3), C-4a (δ 142.7); H-4 (δ 8.40) with C-2 (δ 134.7); H-9 (δ 8.60) with C-7 (δ 132.1); H-8 (δ 8.08) with C-6 (δ 131.7), C-9a (δ 140.8); H-7 (δ 8.30) with C-9 (δ 132.7), C-5a (δ 139.7), C-12 (δ 166.6) clearly assigned the ^{1}H and ^{13}C NMR data of two aromatic rings in compound **2**.

The location of carbomethoxy and carboxamide moieties were determined by ROESY experiment. Main NOE correlations observed from CO–NH_a (δ 9.61) to H-9 (δ 8.60) and CO–NH_b (δ 8.14), from CO–NH_b (δ 8.14) to H-2 (δ 8.70), from $COOCH_3$ (δ 4.01) to H-4 (δ 8.40) and H-7 (δ 8.30) determined the carboxamide and carbomethoxy were linked to C-1 and C-6 positions of compound **2**, respectively. Thus, the structure of compound **2** was elucidated as methyl 6-carbamoylphenazine-1-carboxylate.

The antifungal and antibacterial effects of compounds **1** and **2** were assayed against four root-rot pathogenic fungi of *P. notoginseng* including *Fusarium oxysporum* (ATCC 7808), *Fusarium solani* (ATCC 36031), *Plectosphaerella cucumerina* (local isolate) and *Phoma herbarum* (local isolate) and five pathogenic bacteria including *Staphylococcus aureus* (ATCC 25923), *Staphylococcus albus* (ATCC 10231), *Mycobacterium tuberculosis* (ATCC 25177), *Escherichia coli* (ATCC 25922) and *Bacillus subtilis* (ATCC 6633) using the micro broth dilution method described previously. [6, 7] The minimum inhibitory concentrations (MICs) were defined as the lowest concentration of the antimicrobial agent that completely inhibited the visual growth of an organism. Nystatin and kanamycin were used as positive controls against fungi and bacteria, respectively.

Compound **1** exhibited antifungal activities against *F. oxysporum*, *F. solani*, *P. cucumerina*, and *P. herbarum* with MICs of 32, 64, 32, and 128 μg ml^{-1}, respectively, and antibacterial activities against the *S. aureus*, *S. albus*, and *E. coli* with MICs of 64, 32, 128 μg ml^{-1}, respectively, and was inactive against *M. tuberculosis* and *B. subtilis* at 128 μg ml^{-1}. Compund **2** exhibited antifungal activities against *F. oxysporum, F. solani, P. cucumerina*, and *P. herbarum* with MICs of 64, 64, 128, and 32 μg ml^{-1}, respectively, and antibacterial activities against the *S. aureus, S. albus*, *E. coli*, and *B. subtilis* with MICs of 32, 32, 128, and 64 μg ml^{-1}, respectively, and was inactive against *M. tuberculosis* at 128 μg ml^{-1}.

The phenazine derivatives exhibit a broad range of biological activities, including antibacterial, antimalarial, antitumor, and antiparasitic activities. [8, 9] Although compounds **1** and **2** possessed simple phenazine structures and showed moderate antifungal and antibacterial activities against the pathogenic microorganisms tested in this paper, they were isolated from nature for the first time now. Further investigation on the bioactivity of compounds **1** and **2** need to be undertaken in future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Takahashi Y, Nakashima T. Actinomycetes, an inexhaustible source of naturally occurring antibiotics. Antibiotics. 2018;7:45. https://doi.org/10.3390/antibiotics7020045
2. Barka EA, et al. Taxonomy, physiology, and natural products of actinobacteria. Microbiol Mol Biol Rev. 2016;80:1–43.
3. Li YQ, et al. Diastaphenazine, a new dimeric phenazine from an endophytic *Streptomyces diastaticus* subsp. *Ardesiacus*. J Antibiot. 2015;68:210–2.
4. Abdelfattah MS, Kazufumi T, Ishibashi M. Izumiphenazines A-C: isolation and structure elucidation of phenazine derivatives from *Streptomyces* sp. IFM 11204. J Nat Prod. 2011;73:1999–2002.
5. Chatterjee S, et al. Phencomycin, a new antibiotic from a Streptomyces Species HIL Y-9031725. J Antibiot. 1995;48:1353–4.
6. Tian SZ, et al. Isolation and characterization of new *p*-terphenyls with antifungal, antibacterial, and antioxidant activities from halophilic actinomycete *Nocardiopsis gilva* YIM 90087. J Agric Food Chem. 2013;61:3006–12.
7. Xu XD, et al. Echinosporin antibiotics isolated from *Amycolatopsis* strain and their antifungal activity against root-rot pathogens of the *Panax notoginseng*. Folia Microbiol. 2019;64:171–5.
8. Laursen JB, Nielsen J. Phenazine natural products: biosynthesis, synthetic analogues, and biological activity. Chem Rev. 2004; 104:1663–85.
9. Cimmino A, et al. Phenazines and cancer. Nat Prod Rep. 2012;29:487–501.