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NOTE

Structural elucidation and antimicrobial activity of new phencomycin derivatives isolated from *Burkholderia glumae* strain 411gr-6

Jae-Woo Han[1], Jeong-Do Kim[1], Jung-Min Lee[1], Jong-Hyun Ham[2], Dongho Lee[1] and Beom-Seok Kim[1]

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Burkholderia spp. isolates have been reported to produce antimicrobial compounds, including pyrrolnitrin, pyoluteorin and phenazine compounds.[1–3] Phenazines are colored antimicrobial metabolites that exhibit broad-spectrum antimicrobial activity against bacteria, yeast and filamentous fungi. They comprise a large group of nitrogen-containing heterocyclic compounds with different chemical and physical properties dependent upon the type and position of the functional groups present.[4] In the course of our investigation on the antimicrobial pigments produced by *Burkholderia glumae* strain 411gr-6,[5] we identified phencomycin[6] (a phenazine with two substituents, a carboxyl and a carbomethoxy group; Figure 1) and its new derivatives. Here, we report the purification, structural elucidation and antimicrobial properties of 4-hydroxyphencomycin (**1**) and 5,10-dihydro-4,9-dihydroxyphencomycin methyl ester (**2**; Figure 2).

B. glumae strain 411gr-6 was cross-hatch streaked on 2 kg casamino acid-peptone-glucose (CPG; casamino acid 1 g, peptone 10 g, glucose 10 g and agar 18 g, in 1 L distilled water) agar medium and incubated at 28 °C for 3–4 days. Bacterial cells were harvested and extracted with 2 L MeOH, which had a potent inhibitory effect on spore germination against *Colletotrichum orbiculare*. After concentration *in vacuo*, the residue (6.0 g) was suspended in distilled water and extracted with $CHCl_3$. The antifungal organic layer was evaporated to dryness. The residue (872.0 mg) was applied to Diaion HP-20 resin columns (Mitsubishi Chemical, Tokyo, Japan) and eluted from columns using stepwise gradients of aqueous Me_2CO (0, 20, 40, 60, 80 and 100%, v/v). The antifungal 60% Me_2CO fraction (54.4 mg) was further purified using a semi-preparative Varian Prostar 210 (Palo Alto, CA, USA) HPLC system equipped with a reversed-phase C18 column (ODS-H80, 250 × 10 mm, 4 μm, YMC, Kyoto, Japan). The column was eluted at a flow rate of 2 ml min^{-1} for 30 min using 50% aqueous CH_3CN containing 0.1% formic acid. The effluent was monitored at 365 nm. Final purification using a reversed-phase HPLC system gave phencomycin (0.8 mg, $t_R = 13.8$), **1** (1.0 mg, $t_R = 11.4$) and **2** (2.0 mg, $t_R = 18.4$).

A new phencomycin derivative, 4-hydroxyphencomycin (**1**) was isolated as a yellow powder; UV (MeOH) λ_{max} (logε) 209 (4.61), 270 (4.57), 370 (3.91) nm; ^{1}H NMR ($CDCl_3$, 500 MHz): 14.48 (1H, br s, 11-OH), 9.01 (1H, d, $J = 8.0$, H-2), 8.49 (1H, d, $J = 7.0$, H-7), 8.47 (1H, d, $J = 8.0$, H-9), 8.07 (1H, dd, $J = 7.0$, 8.0), 7.46 (1H, d, $J = 8.0$, H-3), 4.13 (3H, s, 12-OCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): 165.7 (C-11), 165.5 (C-12), 156.7 (C-4), 141.5 (C-2), 140.7 (C-9a and C-10a, overlapped signals), 139.0 (C-5a), 134.4 (C-4a and C-7, overlapped signals), 132.4 (C-9), 132.2 (C-8), 130.5 (C-6), 115.9 (C-1), 110.3 (C-3), 53.1 (12-OCH_3). HR-ESI-MS analysis identified an $[M+H]^+$ ion at *m/z* 299.0675 (calcd *m/z*: 299.0668), consistent with a molecular formula of $C_{15}H_{10}N_2O_5$, one more oxygen than phencomycin.[6] The 12 degrees of unsaturation, implied by the molecular formula and UV absorbance spectrum, were accounted for a phenazine ring substituted by two carbonyl groups. The ^{13}C NMR spectrum of **1** showed resonances for only 13 carbon atoms, because of the overlapped carbon signals at δ 134.4 and 140.7. ^{1}H NMR spectrum of **1** suggested that the three substituents, including a hydroxyl, carboxyl and carbomethoxy group, could be present at 1, 4 and 6, or 1, 6 and 9 positions. The substituents and their location on the phenazine ring were determined by analysis of the ^{1}H–^{1}H COSY and HMBC spectra of **1** (Figure 2).

5,10-Dihydro-4,9-dihydroxyphencomycin methyl ester (**2**) was isolated as a yellow powder; UV (MeOH) λ_{max} (logε) 213 (4.34), 276 (4.78), 370 (3.65) nm; IR (ATR) ν_{max} 3283 (br), 2925 (br), 1644, 1534, 1450, 1393, 1310, 1252, 1055, 834, 667, 553 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): 9.06 (2H, br s, NH), 8.60 (2H, d, $J = 8.0$, H-2 and H-7), 7.32 (2H, d, $J = 8.0$, H-3 and H-8), 4.06 (6H, s, 11-OCH_3 and 12-OCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): 165.5 (C-11 and C-12), 156.3 (C-4, C-9), 139.0 (C-5a and C-10a), 138.3 (C-2 and C-7), 134.7 (C-4a and C-9a), 118.7 (C-1 and C-6), 108.9 (C-3 and C-8), 52.2 (11-OCH_3 and 12-OCH_3). HR-ESI-MS analysis gave an $[M—H]^-$ ion at *m/z* 329.0768 (calcd *m/z*: 329.0765) consistent with a molecular formula of $C_{16}H_{14}N_2O_6$, which indicated 11 degrees of unsaturation.

[1]Department of Biosystems and Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul, Republic of Korea and [2]Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA, USA
Correspondence: Professor Dr B-S Kim, Department of Biosystems and Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, Republic of Korea.
E-mail: biskim@korea.ac.kr
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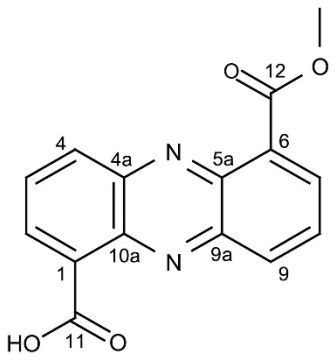

Figure 1 Structure of phencomycin.

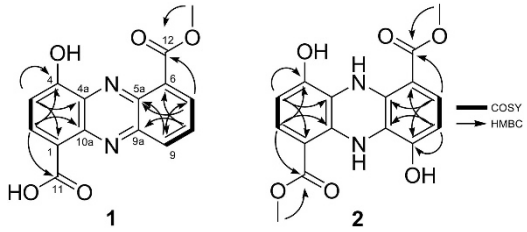

Figure 2 Structures and ^{1}H–^{1}H COSY and key HMBCs of 4-hydroxyphencomycin (**1**) and 5,10-dihydro-4,9-dihydroxyphencomycin methyl ester (**2**).

The ^{13}C NMR spectrum contained only eight signals, two carbomethoxy carbons and aromatic carbons including two secondary and four quaternary carbons, thus indicating that **2** must be a symmetric dimer. The ^{1}H NMR spectrum of **2** showed an NH-proton at δ_H 9.06 (br s), two adjacent aromatic protons at δ_H 8.60 (d, $J = 8.0$ Hz) and 7.32 (d, $J = 8.0$ Hz), and one methoxy signal at δ_H 4.05. The substitution pattern of the individual benzene rings was established by COSY and HMBC (Figure 2). Based on the molecular formula, both parts must be connected by NH bridges forming a 5,10-dihydrophenazine.[7] Comparison of the NMR data of **2** with those of 4,9-dihydroxyphencomycin methyl ester, previously isolated from *Burkholderia cepacia* ATCC 17460,[8–10] revealed a high degree of similarity in the aromatic rings and substituted functional groups except for the presence of two additional identical NH broad singlets at δ_H 9.06. Although antimicrobial pigments with phenazine structures have frequently been isolated from microbial cultures, *N*-unsubstituted simple dihydrophenazine structures are rarely isolated from microbial metabolites.[11] Notably, chlororaphin and dihydrophencomycin methyl ester were identified in cultures of *Bacillus pyocyaneus* and *Streptomyces* sp. B8251, respectively.[11,12] MIC was determined (Table 1) using the Clinical and Laboratory Standards Institute (CLSI) broth micro-dilution susceptibility method (M38-A) in 96-well plates.[13] Compounds were serially diluted by twofold to concentrations of 0, 1, 2, 4, 8, 16, 32, 64 and 128 μg ml^{-1}. Suspensions (1×10^5 spores or mycelial fragments ml^{-1}) of fungi, oomycetes were used as inocula in this test. Phencomycin exhibited weak antibacterial activity and no evident antifungal activity. Compound **1** did not show inhibitory activity against most microorganisms tested in this study, even at concentrations of 128 μg ml^{-1}. The only exception was that 128 μg ml^{-1} of compound **1** inhibited the growth of *Bacillus*. Compound **2** displayed MICs ranging from 1 to 16 μg ml^{-1} against most microorganisms tested. *Bacillus*, *Micrococcus* and *Ralstonia* (MIC values at >128, 128 and 64 μg ml^{-1}, respectively) were less sensitive to compound **2**. In conclusion, antimicrobial pigments produced by *B. glumae* strain 411gr-6 were purified and identified as phencomycin and its derivatives. A novel phencomycin derivative, 5,10-dihydro-4,9-dihydroxyphencomycin methyl ester displayed potent inhibitory activity against a variety of bacteria, yeasts and plant pathogenic fungi. This compound warrants further investigation and could be developed as a broad-spectrum antimicrobial agent.

Table 1 ***In vitro*** **antimicrobial activity of phencomycin derivatives**

	MIC (μg ml^{-1})[a]		
Microorganism	*Phencomycin*	***1***[b]	***2***
Plant pathogenic fungi			
Alternaria brassicicola	>128	>128	2
Aspergillus oryzae	>128	>128	16
Botrytis cinerea	128	>128	1
Cladosporium cucumerinum	>128	>128	4
Colletotrichum gloeosporioides	>128	>128	1
Colletotrichum orbiculare	128	>128	1
Cylindrocarpon destructans	>128	>128	16
Diaporthe citri	128	>128	1
Fusarium oxysporum	>128	>128	16
Magnaporthe oryzae	128	>128	2
Phytophthora capsici	>128	>128	16
Rhizopus stolonifer	>128	>128	16
Sclerotinia sclerotiorum	>128	>128	16
Yeasts			
Candida albicans	>128	>128	2
Saccharomyces cerevisiae	64	>128	1
Bacteria			
Bacillus megaterium	128	128	>128
Escherichia coli	>128	>128	16
Micrococcus luteus	128	>128	128
Pseudomonas syringae	>128	>128	16
Ralstonia solanacearum	>128	>128	64
Xanthomonas campestris	32	>128	2

[a]Concentration that completely inhibits the growth of target microorganism.
[b]4-Hydroxyphencomycin (**1**) and 5,10-dihydro-4,9-dihydroxyphencomycin methyl ester (**2**).

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