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ORIGINAL ARTICLE

6-Hydroxymethyl-1-phenazine-carboxamide and 1,6-phenazinedimethanol from a marine bacterium, *Brevibacterium* sp. KMD 003, associated with marine purple vase sponge

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Two new antibacterial phenazines were isolated from the culture broth of *Brevibacterium* sp. KMD 003 obtained from a marine purple vase sponge of the genus *Callyspongia*, collected in Kyeongpo, Gangwondo, Korea. The structures of these compounds were determined to be 6-hydroxymethyl-1-phenazine-carboxamide (1) and 1,6-phenazinedimethanol (2) through analyses of HR–EI–MS and NMR data. Compounds 1 and 2 showed antibacterial activities against *Enterococcus hirae* and *Micrococcus luteus* with 5 μM MIC values.
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INTRODUCTION

Marine sponges are animals belonging to the phylum porifera and are known to be an abundant source of bioactive secondary metabolites. They are host organisms for various microorganisms, and a number of cytotoxic, anti-microfouling and antimicrobial metabolites have been reported from bacteria associated with marine sponges.[1–4] Marine sponges of genus *Callyspongia* have been shown to contain bioactive secondary metabolites such as pyridine alkaloids,[5] polyacetylenes,[6] peptides,[7] sulfated meroterpenoids[8] and polyketide.[9] However, little is known about the secondary metabolite production of microbes derived from *Callyspongia*. To date, only two cultured microbes, a carotenoid-producing *Streptomyces* sp.[10] and a macrolide-producing fungus *Cladosporium herbarum*,[11] have been isolated from *Callyspongia diffusa* and *C. aerizusa*, respectively.

In an effort to investigate the chemical potential of microbes growing in marine environments, we initiated the investigation of the microbial diversity associated with marine sponges and their secondary metabolites production. During this survey, we isolated a dark green-pigmented bacterium, *Brevibacterium* sp. KMD 003, from the marine purple vase sponge, *Callyspongia* sp. From the liquid culture of KMD 003, we isolated two new antimicrobial phenazines, 6-hydroxymethyl-1-phenazine-carboxamide (**1**) and 1,6-phenazinedimethanol (**2**). Compounds **1** and **2** displayed potent antimicrobial activity against *Enterococcus hirae* and *Micrococcus luteus*.

Phenazine-related compounds have been shown to display a variety of biological activities such as antibiotic, antitumor, antimalaria and antiparasitic activities.[12–15] Phenazines have also attracted great interest due to their physiological roles related to pigment production, quorum sensing and biofilm formation in microbial communities.[16] Here, we report the isolation, structure elucidation and antimicrobial activity of two new phenazines (**1** and **2**) isolated from *Callyspongia*-derived *Brevibacterium* sp.

RESULTS AND DISCUSSION

The sequence analysis of the 16S rRNA gene placed the strain KMD 003 within the genus *Brevibacterium* on the basis of the 99.9 and 99.7% sequence identity with *Brevibacterium sanguinis*[17] and *B. celere*,[18] respectively. The cultured strain KMD 003 was deposited with Korean Culture Center of Microorganisms (KCCM 90080). *B. sanguinis* and *B. celere*, the nearest neighbors of strain KMD 003 in the phylogenetic tree, were isolated from blood cultures of a patient with human immunodeficiency virus and degraded thallus of a brown alga, respectively. A few *Brevibacterium* species have previously been shown to produce the antibiotic phenazine pigments, 1,6-dihydroxyphenazine-5-oxide,[19] 1,6-dihydroxyphenazine-5,10-oxide,[20] iodinin[21] and 1,6-dihydroxyphenazine.[21] However, no biologically valuable organic compounds have been reported from the bacterial strains, *B. sanguinis* and *B. celere*.

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To investigate secondary metabolite production by KMD 003, the strain was cultured in 10 1-l Erlenmeyer flasks each containing 500 ml of TCG liquid medium (total 5 l) ((3 g tryptone (Difco, Sparks, MD, USA), 5 g casitone (Difco) and 4 g glucose (Difco) in 1 l filtered seawater). Metabolite production in the culture broth was monitored daily by HPLC analysis using an Agilent 1100 LC-MS system (Agilent Technologies Inc., Santa Clara, CA, USA) with a Phenomenex Luna 5 C18(2) analytical column (Phenomenex Inc., Torrance, CA, USA) (4.6×150 mm, flow rate 0.7 ml min^{-1}) and a gradient elution of 10–100% acetonitrile in water for 30 min. Two major peaks were observed at retention time 11.3 and 12.2 min on the fourth day of culture. At the end of the culture period (day 7), the cultured broth of the strain KMD 003 was extracted with Amberlite XAD-7 resin (Sigma-Aldrich, St Louis, MO, USA) (20 g l^{-1}) and acetone. The crude extract was subjected to preparative HPLC using C18 column and gradient elution of 10–100% aqueous acetonitrile for the separation of the two major peaks. The subfraction including the major peaks was purified by repeated HPLC using a silica column and an isocratic elution of methylene chloride–methanol (50:1) to afford compounds **1** and **2**.

Compound **1** was isolated as a yellow amorphous powder. The molecular formula was assigned to be $C_{14}H_{11}N_3O_2$ by HR–EI–MS ($(M)^+$=253.0848) (Table 1 and Supplementary Figure S1). The UV spectrum of **1** showing maximal absorptions at 204, 251 and 367 nm was similar to those of the core phenazine structure, which was confirmed by comparison analysis with our in-house HPLC–UV database (Supplementary Figure S2). The ^{1}H NMR spectrum (Supplementary Figure S3) showed six aromatic protons signals at δ_H 8.04 (×2), 8.06, 8.28, 8.43 and 8.68, and one oxymethylene doublet signal at δ_H 5.32. Two-dimensional (2D) NMR analysis, using heteronuclear multiple bond correlation (HMBC) and heteronuclear single quantum coherence (HSQC) experiments, showed that these proton signals correlated with ^{13}C NMR signals at δ_C 132.5, 127.8, 130.6, 127.9, 131.1, 134.5 and 58.8. Other characteristic features of the ^{1}H NMR spectrum of **1** were the presence of a primary alcohol at δ_H 5.49 (1H, t, J=5.0 Hz) and two protons of a primary amide at δ_H 8.12 (1H, br s) and 9.77 (1H, br s). As expected, the amide carbon signal was observed at δ_C 166.1 in the ^{13}C NMR spectrum (Supplementary Figure S4). In addition, the ^{13}C NMR spectrum of **1** showed additional signals attributed to six aromatic quaternary carbons at δ_C 133.7, 140.5, 140.9, 141.5, 141.7 and 142.2. Comprehensive collation of 2D NMR data (Supplementary Figures S5–S7) from ^{1}H-^{1}H COSY, HSQC and HMBC experiments led to the construction of a phenazine structure with a primary amide and a hydroxymethyl functionality. The HMBC correlations between H-2 (δ_H 8.68) and C-1′ (δ_C 166.1), and between 1′-NH (δ_H 8.12) and C-1 (δ_C 131.1) allowed the amide group to be positioned at C-1. The position of the hydroxymethlene group was also determined to be C-6 by the HMBC correlation of a methylene proton signal at δ_H 5.32 with C-6 (δ_C 141.7) and C-7 (δ_C 127.8). In addition, the NOE correlation between 1′-NH at δ_H 9.77 and H-9 (δ_H 8.28), in 2D NOESY experiment with 700 ms mixing time (Supplementary Figure S8), further supported that the functionalities were not at C-1 and C-9, but compound **1** was 1,6-disubstituted phenazines. These data allowed us to assign the structure of compound **1** as 6-hydroxymethyl-1-phenazine-carboxamide (Figure 1).

Compound **2** was obtained as a yellow amorphous powder that was determined to have the molecular formula $C_{14}H_{12}N_2O_2$ by interpretation of HR–EI–MS ($(M)^+$=240.0900) (Supplementary Figure S9) and NMR data (Table 1). The UV and IR spectra (Supplementary Figures S10 and S11) of **2** displayed similar absorption bands with those of compound **1**, indicating that **2** contains a phenazine component. Proton NMR spectral data analysis (Supplementary Figures S12 and S13) indicated signals attributable to a 1,2,3-trisubstitued phenyl group (δ_H 7.95 (1H, dd, J=8.5, 7.0 Hz), 7.98 (1H, br dd, J=7.0, 2.0 Hz) and 8.12 (1H br dd, J=8.5, 2.0 Hz)) and a hydroxymethyl group (δ_H 5.31 (2H, d, J=5.0 Hz) and 5.43 (1H, t, J=5.0 Hz, OH)). The HSQC spectrum (Supplementary Figure S14) showed that these three aromatic protons signals at δ_H 7.95, 7.98 and 8.12 correlated with carbon signals at δ_C 131.2, 127.1 and 128.1, respectively The HSQC spectrum also showed the correlation between the hydroxylmethyl proton signal at δ_H 5.31 and an oxymethylene carbon signal at δ_C 59.5. The ^{13}C NMR spectrum (Supplementary Figure S15) of **2** showed additional signals attributed to three aromatic quaternary carbons at δ_C 140.9, 141.4 and 142.0. Key HMBC correlations allowed the hydroxymethyl group to be positioned at C-1 (δ_C 141.4) (Supplementary Figure S16). Overall analysis of the NMR data for compound **1** indicated a molecular formula of C_7H_6NO, exactly one-half of the molecular formula, $C_{14}H_{12}N_2O_2$, determined by HRMS. Thus, it became clear that compound **1** is a symmetrical phenazine composed

Table 1 The physicochemical and spectral properties of 6-hydroxymethyl-1-phenazine-carboxamide (1) and 1,6-phenazinedimethanol (2)

	1	*2*
Appearance	Yellow amorphous powder	Yellow amorphous powder
Molecular formula	$C_{14}H_{11}O_2N_3$	$C_{14}H_{12}O_2N_2$
HR-EI-MS m/z		
Calcd for	253.0851	240.0899
Found	253.0848	240.0900
UV λ_{max} (CH_3OH) nm (log ε)	204 (4.41), 251 (4.63), 367 (3.95)	203 (4.46), 253 (4.96), 363 (4.11)
IR (neat, $CHCl_3$) ν_{max} cm^{-1}	2922, 2851, 1729, 1452, 1271, 1122, 1072, 854, 805, 752, 699, 617, 575	2923, 2853, 1732, 1667, 1561, 1458, 1380, 1281, 1125, 1073, 746, 699, 617, 575, 544

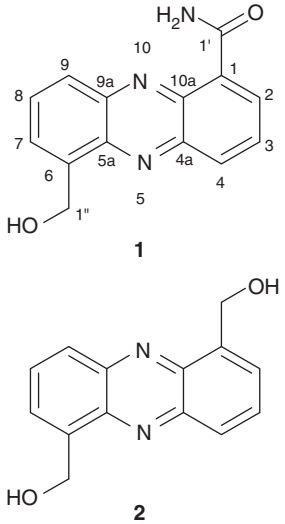

Figure 1 Structures of compounds **1** and **2**.

of two identical 1-hydroxymethyl phenyl groups. The substituted position of two hydroxymehtyl group, 1,6- or 1,9-substitution, was determined to be C-1 and C-6 by NOE correlation between hydroxymethyl (δ_H 5.31) and H-9 signal (δ_H 8.12) in 2D NOESY experiment with 700 ms mixing time (Supplementary Figure S17). These data allowed us to assign the structure of compound **2** as 1,6-phenazinedimethanol (Figure 1).

Although compounds **1** and **2** seem quite simple, small modifications of the core phenazine structure give rise to a dynamic change of color, redox potential and solubility.[16] Moreover, phenazine-1-carboxamide (PCN) and phenazine-1-carboxylic acid (PCA), the structural neighbors of 6-hydroxymethyl-1-phenazinecarboxylic acid (**1**), have important physiological roles, including Fe acquisition,[22] and contribute to pathogen inhibition[23] and microbial biofilm formation.[24] Compound **1** is composed of two functionalities, a hydroxymethyl and a carboxamide, whereas compound **2** includes two hydroxymethyl functionalities, the first examples of these structural compositions. Mono-functionalized phenazine structures including a hydroxymethyl or a carboxamide can be found in 1-phenazinemethanol or PCN. This class of compounds has a variety of ecological functions and therapeutic potential. 1-Phenazinemethanol possesses antifungal activity against *Gaeumannomyces graminis* var. *tritici* and *Pythium* spp.,[25] and PCN displayed antimicrobial activity especially against plant pathogenic Gram-positive bacteria and fungi.[23] PCN is synthesized in *Pseudomonas aeruginosa* by the conversion of PCA in the pyocyanin biosynthetic pathway, which consists of two core loci responsible for the synthesis of PCA and three additional genes encoding unique enzymes involved in the conversion of PCA to pyocyanin, 1-hydroxyphenazine and PCN.[26] In addition, PCN has important physiological roles, including Fe acquisition, due to its redox-activity like other phenazines such as pyocyanin, PCA and 1-hydroxyphenazine *inter alia*.[22] PCA, along with its hydroxylated analog, 2-hydroxy-PCA, contributes to pathogen inhibition and microbial biofilm formation.[24] PCA also increases oxidant formation and alters the expression of IL-8 (interleukin-8) and ICAM-1 (intercellular adhesion molecule-1) in human airway epithelial cells by oxidant-dependent mechanisms.[27]

Compounds **1** and **2** showed antimicrobial activity toward three human pathogenic microorganisms (MIC_{50}=20 μM against *B. subtilis*, 5 μM against *E. hirae* and 5 μM against *M. luteus*). In previously reported literature,[23] the structural neighbors of compound **1**, PCA and PCN, efficiently inhibited the growth of *Bacillus cereus* (MIC <2 μM), whereas showed only modest antimicrobial activity against *M. luteus* (MIC 20 μM). Compounds **1** and **2** were only weakly cytotoxic against eukaryotic cell lines with an IC_{50} value 205.8 and 174.3 μM on HL-60 human leukemia cell lines, respectively, whereas showed no cytotoxicity against five human solid tumor cell lines.

METHODS

General experimental procedures

UV spectra were obtained on an Agilent 8453 UV-Spectrophotometer. FT-IR spectra were measured on a Bruker Tensor27 Spectrophotometer (Bruker, Ettlingen, Germany) at Kangnung-Wonju National University. ^{1}H-, ^{13}C NMR and 2D NMR spectra were obtained in DMSO-d_6 (δ_H 2.55 and δ_C 39.5) on a Varian Unity Plus 500 MHz NMR System (Varian, Palo Alto, CA, USA). Low-resolution ESI–MS was measured on an Agilent Technologies VS/Agilent 1100 system at Kangnung-Wonju National University. HR–EI–MS was measured on a Hewlett-Packard 5890A (Hewlett-Packard, Palo Alto, CA, USA) at the National Center for Inter-University Facilities of Seoul National University. Lichroprep RP-18 (Merck, Darmstadt, Germany, 40–63 μm) was used for column chromatography. A Gilson 321 HPLC system (Gilson, Middleton, WI, USA), with a Delta pak C18 (300×30.00 mm, 15 μm), a Luna C18(2) column (250×10.00 mm, 10 μm) and UV detector (254 nm) was used for preparative HPLC. A Waters 1525 system (Waters, Milford, MA, USA) with PDA detector and a Luna C18(2) column (150×4.6 mm, 5 μm) were used for HPLC analysis.

Isolation of KMD 003 strain, cultivation, extraction

The bacterial strain, *Brevibacterium* sp. KMD 003, was isolated from the tissue of the marine purple vase sponge, *Callyspongia* sp., collected at a depth of 12 m near Kyung-Po beach in Korea (June 2007). The sponge was washed with autoclaved seawater and the tissues ground and diluted with autoclaved seawater (1:10). The diluted suspension (100 μl) was spread on a TCG agar plate. The TCG agar medium contained 3 g tryptone (Difco), 5 g casitone (Difco), 4 g glucose (Difco) and 18 g agar (Difco) in 1 l filtered seawater (pH 7.0). The plate was incubated for 2 weeks at 25 °C under aerobic conditions. KMD 003 was isolated as a dark green-pigmented colony and it was on a TCG agar plate before secondary culturing in TCG liquid medium (25 ml) while shaking at 200 r.p.m. for 7 days at 25 °C. Stocks of the isolated bacterial strain were generated and stored at −80 °C in liquid culture medium containing 15% (v/v) glycerol.

The strain was further cultured in 10 1-l Erlenmeyer flasks each containing 500 ml of TCG liquid medium (total 5 l). The culture flasks were incubated at 25 °C for 7 days with shaking at 200 r.p.m. At the end of the culture period (day 7), Amberlite XAD-7 resin (20 g l^{-1}) was added to each flask, followed by shaking for one additional hour. The resin was collected by filtration through cheesecloth, washed with deionized water and eluted twice with acetone. The acetone solution was then concentrated under reduced pressure to yield 150 mg of crude extract.

Taxonomy

The strain KMD 003 was identified based on 16S rRNA gene sequencing analysis. The chromosomal DNA of strain KMD 003 was extracted using the G-spin Genomic DNA Extraction Kit (iNtRON Biotechnology, Daejoen, Korea). The 16S rRNA gene of strain KMD 003 was amplified by PCR using universal primers 27f and 1492r corresponding to positions 27 in the forward direction and 1492 in the reverse direction of the *Eschrichia coli* 16S rRNA gene.[28] The DNA sequencing reaction was carried out by using an ABI Prism Bigdye terminator cycle sequencing ready reaction kit V.3.1 (Applied Biosystems, Foster City, CA, USA). The PCR cycle-sequencing product was purified by using Montage dye remove kit (Millipore, Bedford, TX, USA) according to the manufacturer's protocol. 16S rRNA gene sequence was determined on a Perkin-Elmer model ABI 3730XL capillary DNA sequencer (Applied Biosystems). The 16S rRNA gene sequence of strain KMD 003 was compared with primary sequence information within the GenBank/EMBL/DDBJ nucleotide sequence database using the BLAST algorithm.[29]

Isolation and purification of phenazines 1 and 2

The crude extract was subjected to preparative HPLC using gradient elution of 10–100% aqueous acetonitrile for 1 h (flow rate 12 ml min^{-1}, Waters Delta pak C18 column 15 μm 300×30 mm) to yield eight subfractions (fractions I–VIII). Fraction VI (33.6 mg) was fractionated by reversed-phase HPLC using a gradient elution of 30–100% aqueous methanol for 1 h (flow rate 4 ml/min, column: Phenomenex Luna C18(2) 10 μm 250×10 mm) to give 15 subfractions (fraction VI-1–VI-15). Compound **1** (5.4 mg) was purified from subfraction VI-9 (9.6 mg) by repeated HPLC using a Phenomenex Luna Silica (2) column (250×10 mm, 10 μm) and an isocratic elution of methylene chloride–methanol (50:1). Compound **2** (5.0 mg) purified from subfraction VI-10 (7.8 mg) was purified by repeated HPLC in the same manner as the purification of compound **1**.

6-Hydroxymethyl-1-phenazine-carboxamide (1)

^{1}H-NMR[a] (500 MHz, DMSO-d_6) δ 5.32. (2H, d, J=5.0 Hz, H$_2$-1″), 5.49 (1H, t, J=5.0 Hz, 1″-OH), 8.04 (2H, m[b], H-8 and H-7), 8.06 (1H, dd, J=8.5, 7.0 Hz, H-3), 8.12 (1H, br s, amide NH$_2$), 8.28 (1H, m[b], H-9), 8.43 (1H, dd, J=8.5, 1.5 Hz, H-4), 8.68 (1H, dd, J=7.0, 1.5 Hz, H-2), 9.77 (1H, br s, amide NH$_2$). [a]The reference for chemical shifts: the signal of DMSO-d_6 (δ_H 2.55). [b]Resonance multiplicity was not able to be assigned due to peak overlapping and second-order effects; ^{13}C NMR[a] (125 MHz, DMSO-d_6) δ 58.8 (C-1″), 127.8

(C-7), 127.9 (C-9), 130.6 (C-3), 131.1(C-1), 132.5 (C-8), 133.7 (C-4), 134.5 (C-2), 140.5 (C-10a), 140.9 (C-5a), 141.5 (C-9a), 141.7 (C-6), 142.2 (C-4a), 166.1 (C-1′). [a]The reference for chemical shifts: the signal of DMSO-d_6 (δ_C 39.5).

1,6-Phenazinedimethanol (2)

^{1}H-NMR[a] (500 MHz, DMSO-d_6) δ 5.31. (4H, d, J=5.0 Hz, H_2-1′ and H_2-1″), 5.43 (2H, t, J=5.0 Hz, 1′-OH and 1″-OH), 7.95 (2H, dd, J=8.5, 7.0 Hz, H-3 and H-8), 7.98 (2H, br dd, J=7.0, 2.0 Hz, H-2 and H-7), 8.12 (2H, br dd, J=8.5, 2.0 Hz, H-4 and H-9); ^{13}C NMR[a] (125 MHz, DMSO-d_6) δ 59.5 (C-1′ and C-1″), 127.1 (C-2 and C-7), 128.1 (C-4 and C-9), 131.2 (C-3 and C-8), 140.9 (C-5a and C-10a), 141.4 (C-1 and C-6), 142.0 (C-4a and C-9a). [a]The reference for chemical shifts: the signals of DMSO-d_6 (δ_H 2.55 and δ_C 39.5).

Antibacterial activity

The antibacterial activity of compounds **1** and **2** were tested in a range of 1.25–10 μg ml^{-1} against six pathogenic microorganisms, *Escherichia coli* (KCTC 2593), *Bacillus subtilis* (KCTC 1021), *Staphylococcus aureus* (KCTC 1916), Methicillin resistance *S. aureus* MRSA 2659, *M. luteus* (KCCM 11548) and *E. hirae* (KCCM 11768). All strains except for *B. subtilis* were grown at 37 °C, and *B. subtilis* was grown at 30 °C in nutrient agar (Difco, USA). Antibacterial activity was determined when the density of the growth control reached an absorbance of 0.150–0.200 at 600 nm. Each pathogenic microorganism was seeded in 96-well plates at 100 μl per well, and incubated for 24 h. Compounds **1** and **2** (1.25, 2.5, 5 and 10 μg ml^{-1}) were then inoculated and incubated in 96-well plates at 30 °C for *B. subtilis* and 37 °C for other pathogens. Growth density was checked every 6 h (0–42 h) at 600 nm.

Cell lines and cytotoxicity

Cytotoxicity was measured by the MTT colorimetric method against HCT116, A549, AGS, MCF-7, HepG2 and HL-60 tumor cell lines. Each cancer cell was seeded in 96-well plates at a density of 10^4 cells per well, and incubated in 5% CO_2 for 24 h at 37 °C. The cells were then treated with varying concentrations (1.56, 3.13, 6.25, 12.5, 25 and 50 μg ml^{-1}) of compounds and incubated in 5% CO_2 for 24 h at 37 °C. After 24 h, cells were incubated in 5% CO_2 for 1 h at 37 °C with 10 μl of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) solution to each well of the plate. Absorbance was then measured at 450 nm using a microplate reader, and experiments were performed in triplicate for each concentration of compounds.

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