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Article

Isolation of secondary metabolites from an Arctic bacterium, *Pseudomonas aeruginosa* and their antimicrobial activities

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북극유래 박테리아, *Pseudomonas aeruginosa*로 부터 대사산물들의 분리 및 항진균 활성

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ABSTRACT: Chemical study of an Arctic bacterium, *Pseudomonas aeruginosa* (Pseudomonadaceae) led to the isolation of two diketopiperazines **1** and **2**, two phenazine alkaloids **3** and **4**, and an indole carbaldehyde **5**, along with a benzoic acid derivative **6**. The structures of the compounds were confirmed by 1D and 2D NMR, and MS experiments, as well as by comparison of their data with published values. Among the isolates, compounds **5** and **6** were isolated for the first time from *P. aeruginosa* of the seawater of Arctic Chuckchi Sea. Antimicrobial activities of compounds **1**–**6** against a *Staphylococcus aureus* and *Candida albicans* were evaluated.

Key words: *Pseudomonas aeruginosa*, antimicrobial activity, diketopiperazine, phenazine, Pseudomonadaceae

Pseudomonas is a diverse genus of Gammaproteobacteria with more than 60 species exhibiting varied life styles in a wide range of environments, including soil, water, plant surfaces, and animals (Moore *et al*., 2006). The genus *Pseudomonas* has had an important role in the biotechnology and pharmaceutical industries due to its metabolic versatility (Gross and Loper, 2009). The vast metabolic diversity of *Pseudomonas* spp. is reflected on the production of a wide variety of bioactive molecules such as mupirocin, fosfadecin, fosfocytosin, and xantholysins (Fuller *et al*., 1971; Gross and Loper, 2009; Buckingham, 2011; Li *et al*., 2013). In particular, *P. aeruginosa* produces a number of toxic substances that have been implicated towards its pathogenicity (Olgerts and Arligues, 1974; David *et al*., 1986; Robert *et al*., 1987). It shows antibacterial, antiarchaeal, antialgal and antifungal activity due to production of various compounds (Hassan and Irwin, 1980; Baron and Rowe, 1981; Dakhama *et al*., 1993; Hernandez *et al*., 2004; Rane *et al*., 2007). The compounds like phenazine serve as electron shuttles in reduction and solubilization of Fe(III) (Hernandez *et al*., 2004), and function as antibiotics (Baron and Rowe, 1981). A previous study reported secondary metabolites, such as diketopiperazines and phenazine derivatives from the Antarctic strain, *P. aeruginosa*, along with their antibacterial activity (Jayatilake *et al*., 1996).

In the course of an ongoing chemical and biochemical studies on the Arctic microorganism, a bacterial strain, *P. aeruginosa* B15H6 displayed potent antimicrobial activity. Although previous research mentioned about metabolites from an Antarctic sponge-associated bacterium, *P. aeruginosa*, this study aims to find other active compounds from an Arctic bacterium, *P. aeruginosa* B15H6. Here, we report the chemical structure elucidation and bioactivity of compounds **1**–**6** isolated from the Arctic bacterium, *P. aeruginosa* B15H6.

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Materials and Methods

Isolation and identification of the bacterium

The bacterial strain (B15H6) was isolated from the seawater of Chukchi Sea in 2012. Seawater (50 μl) was swabbed in freshly prepared SZB medium (glucose 20 g/L, peptone 5 g/L, yeast extract 1 g/L, $FePO_4$ 10 mg/L, and sea water 750 ml/L) and incubated at 25°C for 4 days. Among several selected colonies, a unique bacterial colony with a faint creamy color, and a round thin surface was isolated. The bacterium was identified as *P. aeruginosa* B15H6 by phylogenetic analysis of the 16S rDNA sequence (Baek *et al*., 2015). Stock cultures of the *P. aeruginosa* B15H6 used in this study are preserved at -70°C in KOPRI institute.

Extraction and isolation of the metabolites

The bacterial strain B15H6 was cultivated in 20 L Zobell medium enriched with 18 g/L peptone for 4 days using a 30-L fermenter (Biotron Co.). Cells were removed from the cultures by centrifugation and filtration through a 0.45-μm filter, and the filtrate (20 L) was passed through an Amberlite XAD-20 resin (40 × 8 cm), using a gradient solvent system of H_2O-MeOH (100:0 to 0:100) to give crude extracts (4.5 g). The extracts were diluted with H_2O (1 L) and partitioned using EtOAc (0.5 L × 3 times) to yield EtOAc extracts (1.5 g). A Thin-layer chromatography (TLC) experiment was performed using a solvent mixture, $CHCl_3$-MeOH (9:1), to establish isolation method by silica gel column chromatography. The EtOAc extracts (1.4 g) were separated by column chromatography over silica gel (Column Chromatography, CC; ϕ 10 × 90 cm; 230–400 mesh, 2 kg) using a gradient solvent system of $CHCl_3$-MeOH (99:1 to 50:50), to afford 20 fractions (E1–E20). A fraction combined from E4 to E8 (300 mg), showing similar TLC patterns was subjected to silica gel separation (CC; ϕ 3 cm; 230–400 mesh, 500 g), with $CHCl_3$-MeOH (100:0 to 70:30) as the solvent system, yielding ten subfractions (E4S1 to E4S10). Subfraction E4S5 (70 mg) was chromatographed over a Sephadex LH-20 column and eluted with H_2O-MeOH solvents (90:10 to 100% MeOH), to afford five subfractions (E5S5L1 to E4S5L5). Compounds **1** (10.0 mg) and **2** (8.0 mg) were recrystallized using a pure chloroform ($CHCl_3$), from subfractions E5S5L2 and E5S5L4, respectively. Fraction E10 (100 mg) was chromatographed on a Sephadex LH-20 gel (300 g) column, and eluted with an H_2O-MeOH (90:10 to 0:100) solvent system, to afford five subfractions (E10L1 to E10L5). Subfraction E10L2 was purified by semi-preparative HPLC on a RP-18 column, using MeOH-H_2O mixtures (60:40 to 0:100), to yield **3** (6.0 mg, t_R 85 min) and **4** (5.5 mg, t_R 87 min). Fraction E12 was subjected to semi-preparative HPLC [MeOH/H_2O (50:50 to 0:100)] to yield **6** (2.5 mg, t_R 75 min) and **5** (1.5 mg, t_R 77 min).

Phenazine-1-carboxylic Acid (1) – Yellow needle; UV (MeOH) λ_{max} (log *e*) 365 (3.7) nm; IR ν_{max} (KBr) 3410, 1625 cm^{-1}; LCMS *m/z* 225 $[M + H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) δH 8.98 (1H, dd, *J* = 7.2, 1.6 Hz, H-2), 8.53 (1H, dd, *J* = 8.8, 1.6 Hz, H-4), 8.33 (1H, dd, *J* = 8.8, 1.6 Hz, H-9), 8.28 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 8.04 (1H, overlapping signal, H-3), 8.02 (1H, m, H-7), 8.00 (1H, m, H-8); ^{13}C-NMR (150 MHz, CD_3OD) δC 166.0 (C=O), 125.0 (C-1), 137.5 (C-2), 130.3 (C-3), 135.2 (C-4), 143.5 (C-4a), 139.9 (C-5a), 128.0 (C-6), 133.3 (C-7), 131.8 (C-8), 130.1 (C-9), 144.2 (C-9a), 140.1 (C-10a).

Phenazine-1-carboxamide (2) – Pale yellow needle; UV (MeOH) λ_{max} (log *e*) 365 (3.9) nm; LCMS *m/z* 224 $[M + H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) δH 9.01 (1H, d, *J* = 7.2 Hz, H-2), 8.42 (1H, d, *J* = 8.4 Hz, H-4), 8.28 (1H, dd, *J* = 7.2, 2.4 Hz, H-9), 8.22 (1H, dd, *J* = 7.2, 2.4 Hz, H-6), 7.97 (1H, t, *J* = 7.2 Hz, H-3), 7.92 (1H, m, H-7), 7.92 (1H, m, H-8); ^{13}C-NMR (150 MHz, CD_3OD) δC 166.8 (C=O), 129.0 (C-1), 136.1 (C-2), 130.1 (C-3), 134.5 (C-4), 143.6 (C-4a), 141.6 (C-5a), 129.3 (C-6), 131.9 (C-7), 131.3 (C-8), 129.9 (C-9), 143.3 (C-9a), 140.9 (C-10a).

***cyclo*-(L-Prolyl-L-Phenylalanyl) (3)** – Colorless amorphous powder; $[\alpha]^{20}_D$ –110.5 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log *e*) 254 (3.5) nm; IR ν_{max} (KBr) 3315, 1675, 1420 cm^{-1}; LCMS *m/z* 245 $[M + H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) δH 7.21–7.29 (5H, m, H-1′ to H-6′), 4.44 (1H, br t, *J* = 5.4 Hz, H-9), 4.06 (1H, dd, *J* = 10.8, 6.6 Hz, H-6), 3.53 (1H, ddd, *J* = 20.4, 12.0, 8.4 Hz, H-3b), 3.36 (1H, dddd, *J* = 18.6, 12.0, 7.2, 2.0 Hz, H-3a), 3.16 (2H, m, H-10), 2.08 (1H, m, H-5a), 1.80 (2H, m, H-4), 1.20 (1H, m, H-5b); ^{13}C-NMR (150 MHz, CD_3OD) δC 167.0 (C-1), 46.0 (C-3), 22.9 (C-4), 29.5 (C-5), 60.2 (C-6), 171.0 (C-7), 57.8 (C-9), 38.3 (C-10), 137.4 (C-1′), 129.5 (C-2′ & C-6′), 131.2 (C-3′ & C-5′), 128.2 (C-4′).

***cyclo*-(L-Prolyl-L-Leucine) (4)** − Colorless amorphous powder; $[\alpha]^{20}_{D}$ −120.3 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log *e*) 225 (3.8) nm; LCMS *m/z* 211 $[M+H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) δH 4.26 (1H, t, *J* = 7.2 Hz, H-6), 4.13 (1H, m, H-9), 3.50 (1H, dd, *J* = 13.2, 12.0 Hz, H-3), 2.29 (1H, m, H-5a), 2.04 (1H, m, H-5b), 2.01 (1H, m, H-4a), 1.94 (1H, m, H-4b), 1.93 (1H, m, H-11), 1.89 (1H, m, H-10a), 1.50 (1H, m, H-10b), 0.96 (1H, d, *J* = 6.6 Hz, H-12), 0.95 (1H, d, *J* = 6.0 Hz, H-13); ^{13}C-NMR (150 MHz, CD_3OD) δC 169.0 (C-1), 46.5 (C-3), 23.8 (C-4), 29.2 (C-5), 60.4 (C-6), 172.9 (C-7), 54.7 (C-9), 39.5 (C-10), 25.9 (C-11), 23.4 (C-12), 22.3 (C-13).

1*H*-Indole-3-carbaldehyde (5) − White amorphous powder, LCMS *m/z* 146 $[M+H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) δH 10.07 (1H, s, CHO), 8.32 (1H, br d, *J* = 6.0 Hz, H-4), 7.86 (1H, d, *J* = 3.0 Hz, H-2), 7.45 (1H, br d, *J* = 6.6 Hz, H-7), 7.34 (2H, m, H-5/H6); ^{13}C-NMR (150 MHz, CD_3OD) δC 185.1 (CHO), 135.1 (C-2), 137.0 (C-1a), 124.4 (C-6), 124.0 (C-3a), 123.0 (C-5), 122.0 (C-4), 117.0 (C-3), 111.4 (C-7).

***p*-Hydroxybenzaldehyde (6)** − White amorphous powder, LCMS *m/z* 123 $[M+H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) δH 7.81 (2H, d, *J* = 8.4 Hz, H-2 & H-6), 6.95 (2H, d, *J* = 8.4 Hz, H-3 & H-5); ^{13}C-NMR (150 MHz, CD_3OD) δC 190.7 (CHO), 130.1 (C-1), 132.3 (C-2 & C-6), 115.8 (C-3 & C-5), 161.0 (C-4).

General experimental procedures

Optical rotations were measured on a Rudolph Research Autopol IV multi wavelength polarimeter. UV spectra were recorded on a Shimadzu PharmaSpec-1700 UV-visible spectrophotometer. IR spectra were measured on a Bruker Tensor-27 spectrophotometer. 1D and 2D NMR spectra were recorded on a Bruker AVANCE (600 MHz) spectrometer. Mass spectra and high-resolution MS spectra were performed with a BioTOF II ESI mass spectrometer. Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} (0.25 mm, Merck). Silica gel (230−400 mesh, Merck) and RP-18 (YMC·GEL ODS-A, 12 nm, S-150 μm) were used for column chromatography. Semi-preparative HPLC was conducted on YL9100 HPLC system (Young Lin) equipped with a UV/Vis detector using an Alltech reversed-phase YMC-Pak C-18 column (10 μm, 20 × 250 mm) with a flow rate of 2 ml/min.

Antimicrobial assays

The antimicrobial activities were tested with *Staphylococcus aureus* KCTC 3881 and *Candida albicans* KCTC 27242 (Korean Collection for Type Cultures) in 96-well-plate. Cell culture (95 μl) was distributed in each well, and compounds dissolved in DMSO were added up to final concentrations (0.5, 1, 2, 5, 10, 20, and 50 μM). Total culture (0.1 ml) was incubated at 25°C for 16 h. Cell inhibition was measured at 600 nm using Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific). The IC_{50} value was calculated using exponential trend line in Excel (Microsoft). Kanamycin and nystatin were used as positive controls against the bacterium and yeast, respectively.

Results and Discussion

Repeated chromatography of the culture broth, *P. aeruginosa* on silica gel and YMC-pack C_{18} columns led to the isolation of six compounds (**1**−**6**) (Fig. 1).

Compound **1** was obtained as a yellow needle and its molecular weight was evaluated as *m/z* 225 $[M+H]^+$, by the positive LCMS spectrometry. The UV spectrum showed absorption maxima at 365 nm. The IR spectrum displayed characteristic absorption bands at 3410 and 1625 cm^{-1}, corresponding to the hydroxy group(s) and conjugated C=O group(s), respectively. The ^{13}C NMR spectrum revealed 13 carbon signals, including an ester carbonyl carbon at δC 166.0 (COOH), four N-atom substituted aromatic quaternary carbons at δC 144.2 (C-9a)/143.5 (C-4a)/140.1 (C-10a)/139.9 (C-5a), and an aromatic quaternary

Table 1. Antimicrobial activities of compounds 1−6

Compounds	*S. aureus*	*C. albicans*
	IC_{50} (μg/ml)	IC_{50} (μg/ml)
1	7.17	20.03
2	>50[a]	44.85
3	>50	>50
4	>50	>50
5	13.01	>50
6	>50	>50
Kanamycin[b]	2.84	
Nystatin[c]		1.33

[a] >50, considered to be inactive
[b,c] positive controls for antibacterial and antifungal assays, respectively.

at δC 125.0 (C-1), indicating a characteristic diketopiperazine molecule (Jayatilake *et al*., 1996). The ^{1}H and ^{1}H-^{1}H COSY NMR spectra showed three mutually coupled aromatic proton signals at δH 8.98 (1H, dd, J = 7.2, 1.6 Hz, H-2)/8.53 (1H, dd, J = 8.8, 1.6 Hz, H-4)/8.04 (1H, overlapped, H-3), indicating an 1,2,3-trisubstituted benzene moiety (Fig. 2), and additionally displayed four mutually coupled aromatic proton signals at δH 8.28 (1H, dd, J = 8.0, 0.8 Hz, H-6)/δH 8.02 (1H, overlapped, H-7)/δH 8.00 (1H, overlapped, H-8)/δH 8.33 (1H, dd, J = 8.8, 1.6 Hz, H-9), corresponding to an 1,2-disubstituted benzene moiety. The HMBC spectrum confirmed the position of the carboxylic acid group at C-1 position of 1,2,3-trisubstituted benzene moiety, based on three-bond correlation of H-2 with C-10a and the carbonyl carbon (COOH). The other supporting HMBC correlations were displayed in Fig. 2. Accordingly, compound **1** was assigned as phenazine-1-carboxylic acid, by comparison of their physicochemical data with literature values (Jayatilake *et al*., 1996).

Compound **2** was obtained as a pale yellow needle. The LC-MS spectrum of **2** gave a molecular ion [M + H] at *m/z* 224. The NMR spectra of **2** were quite similar to those of **1**, except for a downfield shifted aromatic quaternary carbon at δC 129.0 (C-1). In addition, the even-numbered mass of **2** indicated that a NH_2 group was attached at carboxylic carbon instead of hydroxy group (-OH) in the carboxylic acid moiety of **1**. Thus, compound **2** was identified as phenazine-1-carboxamide (Jayatilake *et al*., 1996).

Compound **3** was obtained as a colorless amorphous powder. The LCMS spectrum showed a molecular ion peak at *m/z* 245 $[M + H]^{+}$. The ^{1}H NMR spectrum of **3** displayed five mutually coupled aromatic proton signals from δH 7.21 to δH 7.29, indicating 1-substituted benzene moiety. The diketopiperazine skeleton was apparent from the characteristic chemical shifts in ^{1}H- and ^{13}C-NMR spectra; two amides at δC 171.0 (C-1) and 167.0 (C-7) and two N-C residues at [δH 4.06 (1H, dd, J = 10.8, 6.6 Hz)/δC 60.2 (C-6) δH 4.44 (1H, br t, J = 5.4 Hz)/δC 57.8 (C-9)]. Furthermore, the NMR and HSQC spectra of **3** revealed three methylene groups at [δH 3.53 (1H, ddd, J = 20.4, 12.0, 8.4 Hz), 3.36 (1H, dddd, J = 18.6, 12.0, 7.2, 2.0 Hz)/δC 46.0 (C-3), δH 1.80 (2H, m)/δC 22.9 (C-4), and δH 2.08, 1.20 (2H, m)/δC 29.5 (C-5)], all of which confirmed to be closely connected to each other through the HMBC and COSY analyses (Fig. 2). In addition, NMR spectra showed remaining signals for a methylene group at δH 3.16 (2H, m)/δC 38.3 (C-10), displayed two- and three-bond HMBC correlations with C-1/C-2′ (Fig. 2). The relative stereo chemistry of asymmetric carbons was determined as 6*R* and 9*R*, by comparison of the optical rotation, $[\alpha]^{20}_{D}$ − 110.5 (*c* 0.2, MeOH) of **3** with that of the literature value (Adamczeski *et al*., 1995). Accordingly, the structure of **3** was identified as *cyclo*-(L-Prolyl-L-Phenylalanyl) (Adamczeski *et al*., 1995).

Compound **4** was obtained as a colorless amorphous powder. The ^{1}H and ^{13}C NMR spectra of **4** showed similar patterns compare to those of **3**. However, **4** revealed additional signals for a methine group at δH 1.93 (1H, m)/δC 25.9 (C-11) and two symmetrical methyl groups at [δH 0.96 (1H, d, J = 6.6 Hz) /δC 23.4 (C-12), 0.95 (1H, d, J = 6.0 Hz)/δC 22.3 (C-13)] in NMR spectra, indicating an isopropyl group, confirmed the attachment to C-10 position, instead of the benzyl group in **3**. The relative stereo chemistry of **4** was confirmed to be the same with that of **3**. On the basis of the above evidence, **4** was identified as *cyclo*-

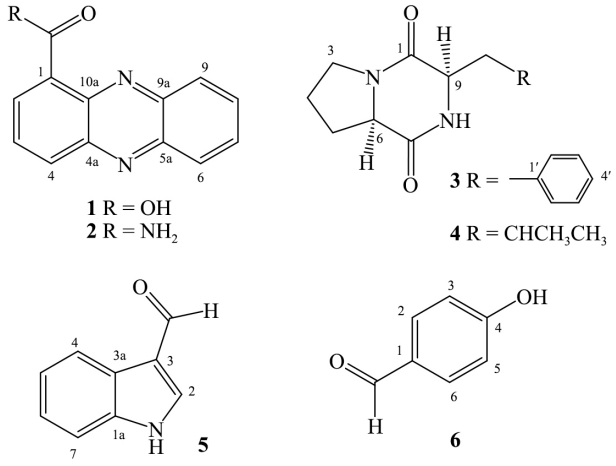

Fig. 1. Structures of compounds 1–6 isolated from *P. aeruginosa*.

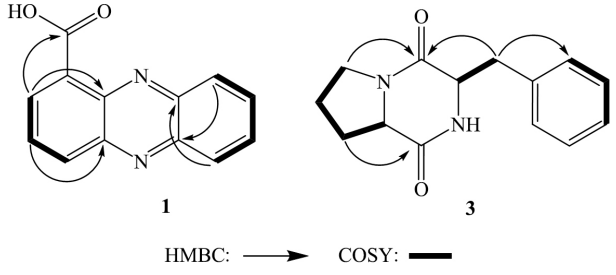

Fig. 2. Important HMBC and COSY correlations of 1 and 3.

(L-Prolyl-L-Leucine), by comparison of their physicochemical data with published values (Adamczeski *et al*., 1995).

Compound **5** was obtained as a white amorphous powder. The LCMS spectrum showed a molecular ion peak at *m/z* 146 $[M + H]^+$. The NMR and HSQC spectra of **5** displayed signals for four mutually coupled aromatic signals at [δH 8.32 (1H, br d, J = 6.0 Hz)/δC 122.0 (C-4), 7.86 (1H, d, J = 3.0 Hz)/δC 135.1 (C-2), 7.45 (1H, br d, J = 6.6 Hz)/δC 111.4 (C-7), δH 7.34 (1H, m)/δC 123.0 (C-5), and δH 7.34 (1H, m)/δC 124.4 (C-6)], a tri-substituted olefinic group at [δH 7.86 (1H, d, J = 3.0 Hz)/δC (138.0, C-2) and δC (117.0, C-3)], an aromatic quaternary carbon at δC 124.0 (C-3a), and a downfield shifted aromatic quaternary carbon at δC 137.0 (C-1a) due to a nitrogen atom attachment, implying the presence of an indole skeleton. Furthermore, the ^{1}H and ^{13}C NMR spectra revealed downfield shifted signals at δH 10.07 (1H, s)/δC 185.1 (CHO), indicating an aldehyde group. The HMBC spectrum displayed two- to three-bonds correlations from H-2 to the aldehyde carbon (CHO)/C-3a and from H-4 to C-3/C-1a/C-3a (Fig. 2), confirmed the identification of compound **5** as 1*H*-indole-3-carbaldehyde (Wang *et al*., 2013).

The ^{1}H and ^{13}C NMR spectra of **6** indicated a benzoic acid derivative, which were in good agreement with those of *p*-hydroxybenzaldehyde (**6**) (Kim *et al*., 2003). To the best of our knowledge, compounds **5** and **6** were isolated for the first time from *P. aeruginosa* of the seawater of Arctic Chuckchi Sea.

The EtOAc-soluble extract from the culture broth of *P. aeruginosa* B15H6 exhibited potent antimicrobial activity against *Candida albicans* and *Staphylococcus aureus*, respectively (data not shown).

The secondary metabolites, **1**–**6** isolated from the extract were also evaluated for their antimicrobial activity. Among the isolates, compound **1** displayed potent antibacterial activity with an IC_{50} value of 7.17 μM against the Gram-positive bacteria, *S. aureus*, and also showed moderate antiyeast activity with an IC_{50} value of 20.03 μM against *C. albicans*; the indol alkaloid **5** exhibited moderate antibacterial activity (IC_{50} value, 13.01 μM) against *S. aureus*. Meanwhile the phenazine amide **2** showed only weak inhibitory activity (IC_{50} value, 44.85 μM) against *C. albicans*. Other remaining compounds were inactive. In study of antibacterial activities for phenazine alkaloids, all compounds of the phenazine class have been reported to have antibacterial properties (Dahiya *et al*., 1988; Dakhama *et al*., 1993; Jayatilake *et al*., 1996). These results suggest the phenazine carboxylic acid **1** could possess antimicrobial activity.

적 요

북극 유래 박테리아인 *Pseudomonas aeruginosa* 균주의 대사산물에 대한 화학적 연구는 벤조산 유도체 **6**번을 포함하여, 두 개의 diketopiperazine **1**과 **2**, 두 개의 phenazine alkaloid **3**과 **4**, indole carbaldehyde **5**번을 분리하였다. 화합물들의 구조는 1D 과 2D NMR, 및 MS 기법들과 기존 보고된 문헌 값 과의 비교에 의하여 동정되었다. 분리된 화합물들 중 **5**번과 **6**번은 북극 척지해 해수의 *P. aeruginosa*로 부터 처음으로 보고 되었다. 대사산물들 **1**-**6**의 항균 활성은 *Staphylococcus aureus*과 and *Candida albicans*에 대하여 측정하였다.

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