Izumiphenazine D, a New Phenazoquinoline *N*-Oxide from *Streptomyces* sp. IFM 11204

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Received December 9, 2010; accepted January 24, 2011; published online January 25, 2011

A new phenazine derivative named izumiphenazine D (1), together with three known metabolites, 1-hydroxyphenazine (2), phenazine-1-carboxylic acid (3) and 6-hydroxyphenazine-1-carboxylic acid (4) has been isolated from the ethyl acetate extract of culture of *Streptomyces* sp. IFM 11204. The structure of 1 was established *via* spectroscopic methods, including 1D- and 2D-NMR measurements.

Key words *Streptomyces* sp.; phenazine; spectroscopy; *N*-oxide

Actinomycetes from soil and marine source are widely recognized to produce secondary metabolites including a number of antimicrobials as streptomycin, erythromycin, and tetracycline with original and ingenious structures and potent biological activities.[1)] Therefore, it is thought that actinomycete is a potential resource for new lead or seed compounds in the drug development. In the course of our screening program for new antibiotics from actinomycete strains,[2—4)] we found that *Streptomyces* sp. IFM 11204 is a prolific source of highly pigmented aromatic compounds, which stimulated our interest in identifying their structures and evaluating their biological activity. Recently, we isolated new phenazine derivatives named izumiphenazines A—C.[5)] Further investigation of the culture of *Streptomyces* sp. IFM 11204 resulted in the isolation of another phenazine metabolite named izumiphenazine D (**1**) (Fig. 1). Herein we describe the isolation and structural elucidation of this new compound. The new phenazine derivative (**1**) was evaluated for the Wnt signal inhibitory activity, since we are recently interested in screening studies targeting signaling molecules related to cancer diseases.[6)]

Results and Discussion

With a well-grown agar culture of the terrestrial *Streptomyces* sp. IFM 11204, four 500-ml Sakaguchi flasks each containing 100 ml of liquid Waksman medium[7)] were inoculated and incubated for 5 d at 28 °C while shaking at 200 rpm. The seed culture (10 ml) was used to inoculate twenty 3-l flasks each containing 700 ml of the same medium, which were incubated using similar conditions. After harvesting, the culture broth (14 l) was centrifuged at 3500 rpm for 20 min. The resulting mycelial cake was extracted three times with acetone. After removal of acetone, the aqueous solution was extracted three times with EtOAc. The EtOAc-soluble portion was concentrated under reduced pressure. The culture broth was extracted three times with EtOAc. The crude extract was applied to silica gel flash column chromatography under gradient of $CHCl_3$/MeOH and separated into 6 fractions. Further purification of these fractions on octadecylsilane (ODS) column chromatography, preparative thin layer chromatography (PTLC) and Sephadex LH-20 yielded three known and one new natural products. Compounds **2**—**4** were all obtained as yellow solids. Searching in SciFinder with the information from NMR and mass data led to the identification of these compounds as 1-hydroxyphenazine (**2**),[8)] phenazine-1-carboxylic acid (**3**)[9)] and 6-hydroxy-phenazine-1-carboxylic acid (**4**).[10)] All of the known compounds were identified by comparison of the NMR data with the literature values.

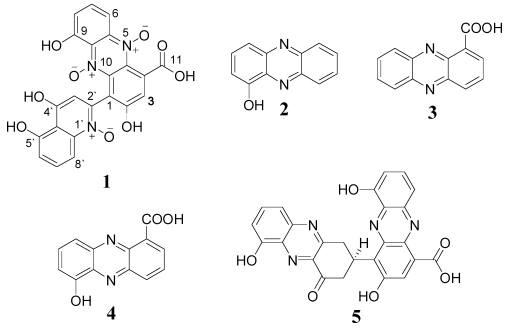

Fig. 1. Structure of Isolated Compounds

Izumiphenazine D (**1**) was isolated as yellow solid. On the TLC plate after developing compound **1**, it gave a positive color reaction after spraying with Dragendorff's reagent and dark fluorescence under UV light at 254 nm. The molecular weight of **1** was determined to be 463 Daltons from the negative ion mode of electrospray ionization (ESI) mass spectra (*m*/*z* 462 $[M-H]^-$). The molecular formula was determined as $C_{22}H_{13}N_3O_9$ by high resolution (HR)-ESI-MS *m*/*z* 462.0816 $[M-H]^-$ (Calcd 462.0784, Δ +3.2 mmu). The UV spectrum of **1** (maxima were visible at 451, 384, 272 nm) resembles the UV spectra of many phenazine derivatives.[11)] Absorption bands at 3358 and 1720 cm^{-1} in the IR spectrum suggested the presence of hydroxyl and carbonyl groups, respectively. The ^{1}H-NMR spectrum of **1** in DMSO-d_6 (Table 1) exhibited five D_2O exchangeable protons [δ_H 14.20 (1H, br s), 10.36 (1H, br s), 10.18 (1H, br s), 9.81 (1H, br s), and 8.31 (1H, br s)]. The aromatic region of **1** showed two sharp singlets [δ_H 7.92 (1H, s), 7.41 (1H, s)] and two 1,2,3-trisubstituted aromatic spin systems [δ_H 7.73 (1H, t, J=8.1 Hz), 7.68 (1H, d, J=8.1 Hz), 7.13 (1H, d, J=8.1 Hz), 7.81 (1H, t, J=7.6 Hz), 7.70 (1H, d, J=7.6 Hz), 7.19 (1H, d, J=7.6 Hz)]. All the assignments were clearly derived from 2D-NMR spectra (^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC)). The

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Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for Izumiphenazine D (**1**) in DMSO-d_6

Position	δ_H (ppm)	δ_C (ppm)	HMBC (^{1}H to ^{13}C)
1	—	132.6	—
2	—	154.8	—
3	7.41 s	113.6	1, 2, 11
4	—	130.3	—
4a	—	136.8	—
5a	—	139.7	—
6	7.68 d (8.1)	118.9	8
7	7.73 t (8.1)	131.7	5a, 9
8	7.13 d (8.1)	110.4	6
9	—	159.8	—
9a	—	138.3[a)]	—
10a	—	132.2	—
11	—	166.7	—
2′	—	140.5	—
3′	7.92 s	122.4	4′, 4a′, 1
4′	—	150.8	—
4a′	—	132.4[a)]	—
5′	—	154.3	—
6′	7.19 d (7.6)	108.8	4a′, 5′, 8′
7′	7.81 t (7.6)	132.3[b)]	5′, 8′a
8′	7.70 d (7.6)	119.6	4a′, 6′
8a′	—	143.3	—
2-OH	8.31 br s	—	—
9-OH	10.36 br s	—	—
4′-OH	9.81 br s	—	—
5′-OH	10.18 br s	—	—
11-OH	14.20 br s	—	—

a) Values were observed from HMBC. *b*) Values were observed from HMQC.

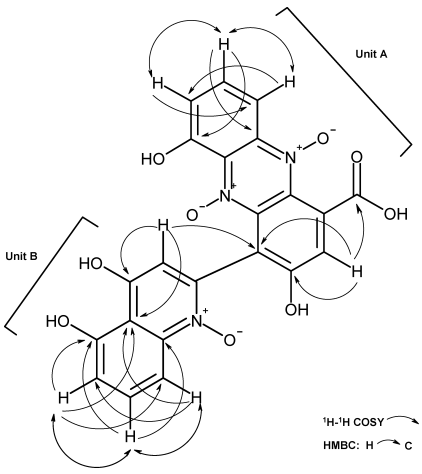

Fig. 2. Structure of Compound **1** with Key 2D-NMR Correlations

^{13}C-NMR spectrum of **1** revealed one carbonyl (δ_C 166.7), four oxygenated sp^2 carbons (δ_C 159.8, 154.8, 154.3, 150.8), eight sp^2 methine carbons (δ_C 132.3, 131.7, 122.4, 119.6, 118.9, 113.6, 110.4, 108.8) and nine quaternary carbons. Searching in SciFinder with the molecular formula and NMR data led to a new compound.

Analysis of the 2D-NMR spectra (^{1}H–^{1}H COSY, HMQC and HMBC) of izumiphenazine D (**1**) gave two units A and B (Fig. 2). Two ABC systems in **1** were constructed by interpretation of the COSY spectrum. The HMBC correlations of H-6 (δ_H 7.68) with C-8; H-7 (δ_H 7.73) to C-5a and C-9; and H-8 (δ_H 7.13) to C-6 supported this assumption in unit A. Correlations of H-6′ (δ_H 7.19) with C-4′a, C-5′, and C-8′; H-7′ (δ_H 7.81) to C-5′ and C-8′a; and H-8′ (δ_H 7.70) to C-4′a, C-6′ also confirmed 1,2,3-trisubstituted benzene ring in unit B. Moreover, correlation of the aromatic proton H-3 (δ_H 7.41) with C-1, C-2 and C-11 allowed the assignment of *m*-hydroxybenzoic acid in unit A. Several correlation patterns derived from HMBC and COSY data of **1** were found to be identical to those of izumiphenazine B (**5**).[5)] The HMBC couplings of H-3′ (δ_H 7.92) with C-4′ and C-4a′, along with ^{13}C and ^{1}H shifts in unit B, pointed to a 2′-substituted 4′,5′-dihydroxy quinoline *N*-oxide. The proton signal at δ_H 7.92 was established as H-3′ in a quinoline and not in an isoquinoline, as the low-field ^{13}C shift of both C-3′ and C-2′ (δ_C 122.4, 140.5, respectively) cannot be explained by the latter. Connectivity between units A and B was confirmed from the HMBC correlations of both H-3′ (δ_H 7.92) and H-3 (δ_H 7.41) with the quaternary carbon δ_C 132.6 (C-1). By combination of the above evidences and presence of nine oxygen atoms in the molecule, it was concluded the structure of izumiphenazine D (**1**) as in Fig. 1. Several phenazines *N*-dioxide such iodinine and lomondomycin were isolated from different actinomycetes.[12)] On the other hand, quinoline *N*-oxide alkaloids like KF-8940[13)] and aurachin A[14)] are particularly abundant in nature and have been isolated from several microbial sources. To the best of our knowledge, izumiphenazine D (**1**) is the first example of microbial phenazine natural products containing quinoline-*N*-oxide moiety.

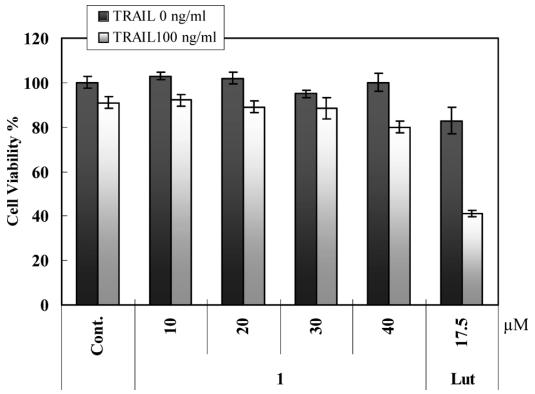

Fig. 3. Effect of Compound **1** and Luteolin (Positive Control: Lut) in the Presence and Absence of TRAIL on the Viability of AGS Cells

The standard error bar represents the means (n=3±S.D.).

The bioactivity of compound **1** was evaluated for effects on TNF-related apoptosis inducing ligand (TRAIL)-resistance in gastric adenocarcinoma (AGS) cells by comparing cell viability in the presence and absence of TRAIL (100 ng/ml) using the fluorometric microculture cytotoxicity assay (FMCA) method.[15)] As shown in Fig. 3, treatment with 100 ng/ml of TRAIL for 24 h resulted in only a slight decrease in cell viability (91±4.5%), while luteolin[16)] (a positive control) produced about 40% more inhibition along with TRAIL than the agent alone at 17.5 μM. With 40 μM of **1**, cell viability was 99%, whereas the same concentration of **1** in the presence of 100 ng/ml TRAIL reduced cell viability to 80% of control levels, which was 19% lower than the agent alone. These results suggest that izumiphenazine D (**1**) had a synergistic effect in combination with TRAIL against AGS cells. We also examined the Wnt signal inhibitory activity of the

izumiphenazine D (**1**) using a luciferase reporter gene assay in SuperTOP-Flash transfected cells.[17] The results showed that compound **1** did not exhibit inhibition of Wnt signal transcription activity even at 50 μM.

Experimental

General Experimental Procedures The NMR data were measured on a JEOL JNM ecp600 spectrometer. Mass spectra were recorded on AccuTOF-T100LP (JEOL) mass spectrometer. IR spectra were recorded on attenuated total reflection (ATR) in a Jasco FT-IR 230 spectrophotometer, and UV spectra were obtained on a Shimadzu UV mini-1240 spectrometer.

Microbial Strain *Streptomyces* sp. IFM 11204 was separated from a soil sample collected from Izumi forest in Chiba city, Japan. The identification was carried out by Professor Tohru Gonoi at the Medical Mycology Research Center, Chiba University, where a voucher specimen is deposited with code IFM 11204.

Extraction and Isolation The culture broth (14 l total), obtained as described previously,[5] was harvested and centrifuged to separate the mycelia and supernatant. The pH of the culture broth was adjusted to 5.0 by acetic acid and then extracted four times with EtOAc. The mycelia were extracted three times with acetone. After removal of acetone, the aqueous solution was extracted four times with EtOAc. As the TLC of both extracts from the culture filtrate and mycelia showed the same composition, they were combined and concentrated under reduced pressure. The crude extract of *Streptomyces* sp. IFM 11204 (5.21 g) was subjected to silica gel 60N column chromatography (CC) under gradient of $CHCl_3$/MeOH to afford six fractions. Fraction II was subjected several times to Sephadex LH-20 ($CHCl_3$/MeOH, 3 : 2) to obtain compound **2** (5.8 mg). Fraction III was subjected to ODS CC followed by Sephadex LH-20 (MeOH) to get compounds **3** (3.6 mg) and **4** (6.8 mg). Compound **1** (3.1 mg) was isolated from fraction IV by precipitation from acetone solution and finally purified by PTLC (5 plates, 20×20 cm, $CHCl_3$/20%MeOH).

Izumiphenazine D (**1**): Yellow solid; UV λ_{max} (MeOH) 272 (ε 15500), 348 (6600) and 451 (6000) nm; IR (ATR) ν_{max} 3358, 1720, 1559, 1398, 1085 and 750 cm^{-1}; ^{1}H- and ^{13}C-NMR (Table 1); Negative ESI-MS *m/z*: 462.0 $[M-H]^-$; HR-ESI-MS *m/z*: 462.0816 (Calcd for $[C_{22}H_{12}N_3O_9]^-$, 462.0784).

Cell Cultures AGS cells were derived from the Institute of Development, Aging and Cancer, Tohoku University. The cells were cultured in RPMI-1640 medium (Wako, Osaka, Japan) with 10% fetal bovine serum (FBS) and maintained in a humidified incubator at 37 °C in 5% CO_2/95% air.

TRAIL-Resistance Test TRAIL-resistance was assessed by comparison of cell viability in the presence and absence of TRAIL with TRAIL-resistant human AGS cell lines.[4,18] AGS cells were seeded in a 96-well culture plate (6×10^3 cells per well) in 200 μl of RPMI medium containing 10% FBS. Cells were incubated at 37 °C in a 5% CO_2 incubator for 24 h. Then the test samples with or without TRAIL (100 ng/ml) at different doses were added to each well. After 24 h of incubation, the cells were washed with phosphate buffered saline (PBS), and 200 μl of PBS containing fluorescein diacetate (10 μg/ml) was added to each well. The plates were then incubated at 37 °C for 1 h, and fluorescence was measured in a 96-well scanning spectrofluorometer at 538 nm with excitation at 485 nm.

Acknowledgments This work was supported by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS). The research described in this paper was supported by JSPS postdoctoral fellowship (ID No. P09042).

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7) Liquid Waksman medium (100 ml): glucose (2 g), meat extract (0.5 g), peptone (0.5 g), dried yeast (0.3 g), NaCl (0.5 g), and $CaCO_3$ (0.3 g); agar (1.5 g) was added for solidification.
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