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Glionitrin B, a Cancer Invasion Inhibitory Diketopiperazine Produced by Microbial Coculture

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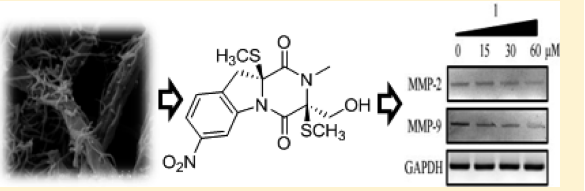
Supporting Information

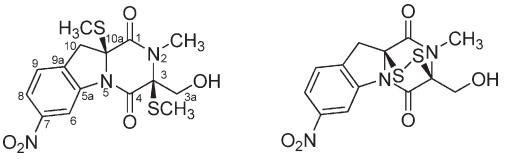
ABSTRACT: A new diketopiperazine, glionitrin B (**1**), was produced using a microbial coculture of the fungus *Aspergillus fumigatus* KMC-901 and the bacterium *Sphingomonas* sp. KMK-001 that were isolated from acidic coal mine drainage. The structure of **1** was determined to be (3*S*,10a*S*)-dithiomethylglionitrin A. This structure was determined by the analyses of extensive NMR data and the circular dichroism spectra of the natural product and a semisynthetic compound derived from glionitrin A. In contrast to glionitrin A (**2**), glionitrin B (**1**) is not cytotoxic against the human prostate cancer cell line DU145. However, compound **1** caused suppression of DU145 cell invasion, producing 46% inhibition at 60 μM.



Microbial products have provided many novel chemical scaffolds for drug discovery. However, the reisolation of known compounds is becoming increasingly problematic for the discovery of new pharmacophores. One potential approach to reduce this problem is the use of a microbial coculture. The competition that results from coculturing two different microorganisms may induce an unexpressed pathway for the biosynthesis of new secondary metabolites. Coculture investigations in the natural products chemistry field have attempted to gain incremental yields of previously described secondary metabolites, mainly antibiotics. However, microbial coculture studies that aim to produce new chemical structures and induce gene expression are in their early stages. To date, approximately 11 new bioactive microbial constituents have been reported in microbial coculture publications.[1,2]

Our recent investigation of new bioactive secondary metabolites in a microbial coculture medium using *Aspergillus fumigatus* and *Sphingomonas* sp. led to the isolation of a cytotoxic diketopiperazine disulfide, glionitrin A (**2**),[3] and the detection of a small amount of another unknown diketopiperazine. During the investigation to identify the minor diketopiperazine, we observed that the production of this minor compound, named glionitrin B (**1**), was dramatically increased during long-term coculture experiments (longer than 18 days) or when the bacterial growth was dense in the coculture medium. In the present paper, we report the isolation procedure, structure elucidation, and biological activity of glionitrin B (**1**).

Sphingomonas sp. strain KMK-001 and *Aspergillus fumigatus* strain KMC-901 were isolated from acidic mine drainage that was collected at the abandoned Young-dong coal mine. The *Sphingomonas* sp. KMK-001 was cultured in Czapek-Dox liquid medium, and after 48 h, *Aspergillus fumigatus* strain KMC-901 was inoculated into the same medium. The broth was extracted with ethyl acetate after 18 days of incubation. The successive flash and HPLC separations were conducted to afford glionitrin B (**1**) from the ethyl acetate soluble fraction.



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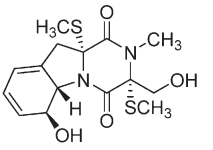

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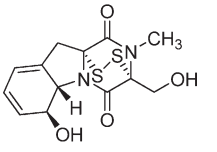

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Glionitrin B (**1**) was isolated as an optically active, yellowish, amorphous gum ($[\alpha]_D^{25}$ −7.7, *c* 0.10, MeOH). The molecular formula was determined to be $C_{15}H_{18}N_3O_5S_2$ using high-resolution FABMS data. The presence of strong IR absorption bands at 3447 and 1674 cm^{-1} suggested the presence of hydroxyl and carbonyl functional groups, respectively. The ^1H NMR spectrum of **1** in deuterated methanol exhibited nine apparent proton signal resonances, including three aromatic protons (δ_H 8.83, 8.14, and 7.62), two oxymethylene protons (δ_H 4.39 and 3.98), one singlet methylene proton (δ_H 3.73), one *N*-methyl proton (δ_H 3.20), and two *S*-methyl protons (δ_H 2.36 and 2.27).

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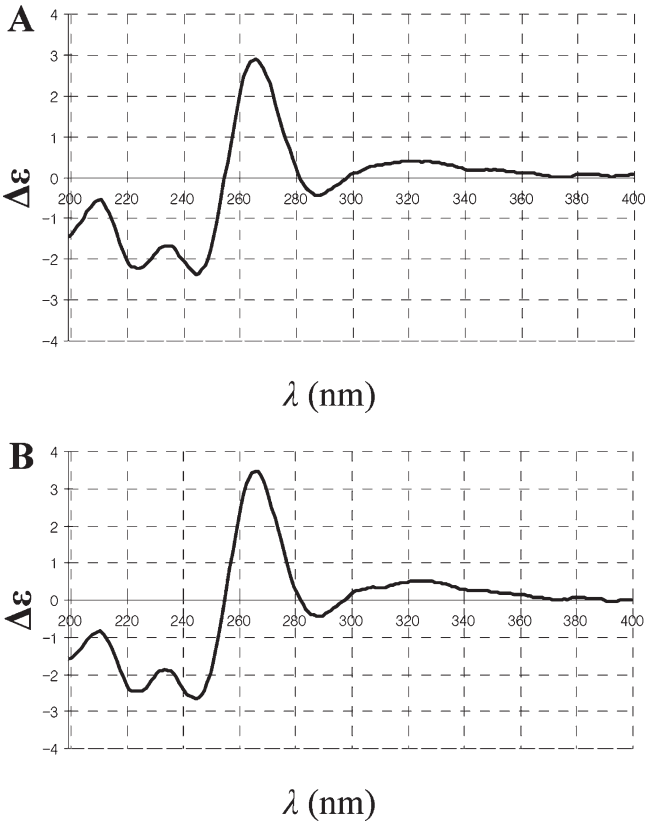

Figure 1. Circular dichroism (CD) spectra of natural **1** (A) and semisynthetic **1** (B).

Subsequently, the analysis of the ^{13}C NMR and gHSQC spectra of **1** indicated the presence of 15 carbons, comprising two amide carbonyl carbons (δ_C 166.1 and 163.0), five quaternary carbons (δ_C 148.0, 141.9, 137.3, 72.4, and 71.8), three aromatic methine carbons (δ_C 125.9, 121.1, and 112.2), one oxygenated methylene carbon (δ_C 63.9), one methylene carbon (δ_C 39.2), one *N*-methyl carbon (δ_C 28.1), and two *S*-methyl carbons (δ_C 13.2 and 12.2). The ^{1}H and ^{13}C NMR spectroscopic data of **1** were very similar to those of glionitrin A (**2**), with the exception of two *S*-methyl signals (δ_H 2.36 and δ_C 12.2 vs δ_H 2.27 and δ_C 13.2). The ^{13}C chemical shift of C-7 (δ_C 148.0)[4] and the IR absorption bands[5] at 1526 and 1347 cm^{-1} indicated that glionitrin B (**1**) contained a nitro functionality. The substituted positions of the two *S*-methyl groups were determined as C-3 and C-10a on the basis of the key HMBC correlations from a *S*-methyl proton at δ_H 2.36 to a quaternary carbon at δ_C 72.4 and from a *S*-methyl proton at δ_H 2.27 to a quaternary carbon at δ_C 71.8 (see Figure S1 thru S14 in the Supporting Information for the spectral data of **1**).

The absolute configuration at C-3 and C-10a in **1** was determined by the comparison of the circular dichroism spectrum of **1** with that of a semisynthetic compound derived from the reduction and methylation of glionitrin A (**2**).[6] Treatment of compound **2** with sodium borohydride and iodomethane in methanol yielded **1** (3*S*,10a*S*-dithiomethylglionitrin A). The circular dichroism spectrum of this compound was identical to that of **1**. The CD spectra exhibited a negative Cotton effect near λ 220 nm and λ 240 nm and a strong positive Cotton effect at λ 265 nm (Figure 1). Therefore, the absolute configuration of the chiral centers at C-3 and C-10a in **1** was determined to have the same configuration as glionitrin A (**2**), 3*S* and 10a*S* (see Figure S15 and S16 for the ^{1}H and ^{13}C NMR spectra of a semisynthetic compound **1**).

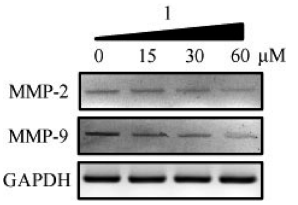

Figure 2. Effects of glionitrin B (**1**) on the mRNA levels of MMP-2 and MMP-9 in DU145 cells. The mRNA levels of MMP-2 and MMP-9 were determined using RT-PCR. GAPDH was used as an internal control.

Natural 2,5-diketopiperazines are well known.[7−9] The majority are biosynthesized from the condensation between two amino acid precursors via the catalytic control of nonribosomal peptide synthetases or the recently reported cyclodipeptide synthases.[10−14] The structural diversity of these diketopiperazines is achieved through subsequent reactions, such as cyclization,[15] prenylation,[16] and dimerization.[17,18] 2,5-Diketopiperazine derivatives have a wide range of biological activities, including as modulators of plasminogen activator inhibitor (PAI)-1,[19] platelet activating factor antagonists,[20] cell cycle inhibitors,[21] tryptase inhibitors,[22] antifouling activities,[23] and oxytocin antagonists.[24]

In the present study, the diketopiperazine disulfides glionitrin A (**2**) and gliotoxin (**4**) exhibited significant cytotoxicity against a DU145 human prostate cancer cell line, whereas glionitrin B (**1**) and **3** did not effect DU145 cell viability (Figure S18). However, glionitrin B (**1**) reduced the invasion of DU145 cells in a concentration-dependent manner (Figure S19). To evaluate the effect of the dithiomethyl functionality on the invasive capability of DU145 cells, we prepared 3*R*,10a*R*-dithiomethylgliotoxin (**3**) through the reduction and methylation of **4**. The anti-invasion effects of compounds **1** through **4** were evaluated using the same bioassay conditions for all four compounds. As expected, glionitrin A (**2**) and gliotoxin (**4**) did not exhibit anti-invasion effects at a noncytotoxic concentration (0.2 μM). In addition, 3*R*,10a*R*-dithiomethylgliotoxin (**3**) exhibited less effect on the invasion of DU145 cells, despite the fact that **3** contained the same dithiomethyl group as **1**. The treatment of 60 μM glionitrin B (**1**) for 24 h led to 46% inhibition of the invasive capability of DU145 cells compared to an untreated group, whereas **3** (60 μM) exhibited 22% invasion inhibition (Figure S19).

Cancer cell invasion through the extracellular matrix (ECM) is an important step in tumor metastasis.[25] The invasive ability of tumor cells is related to an overexpression of proteolytic enzymes, such as MMP-2, MMP-9, and u-PA. During the invasion process, these proteolytic enzymes degrade ECM components, forming the basal membrane that surrounds blood vessels to allow cancer cells to invade.[26] To identify the underlying mechanism of the anti-invasive activity of glionitrin B (**1**), we investigated changes in MMP-2 and MMP-9 expression using RT-PCR. As shown in Figure 2, glionitrin B (**1**) led to a decrease in MMP-2 and MMP-9 mRNA levels in a concentration-dependent manner.

The tendency of prostate cancer toward skeletal metastases is well known, and the majority of patients who die from prostate cancer have metastatic bone disease.[26] Therefore, the inhibition of metastasis is important for successful cancer treatment. The results of our research suggest that glionitrin B may be developed as an antimetastatic agent in cancer treatment.

Table 1. NMR Spectral Data for 1 in Methanol-d_4 (δ_H 3.32, δ_C 47.6)

position	δ_C[a]	δ_H[b]	mult (*J* in Hz)		gCOSY	gHMBC	1D NOE
1	166.1			C			
2-Me	28.1	3.20	s	CH_3		C1, C3	3a$_\beta$
3-SMe	12.2	2.36	s	CH_3		C3	3a$_\alpha$
3	72.4			C			
3a$_\alpha$	63.9	4.39	d (12.0)	CH_2	3a$_\beta$	C4	3-SMe
3a$_\beta$		3.98	d (12.0)		3a$_\alpha$	C3, C4	2-Me, 3-SMe
4	163.0			C			
5a	141.9			C			
6	112.2	8.83	d (2.5)	CH	8	C5a, C7, C8, C9a	
7	148.0			C			
8	121.1	8.14	br dd (8.5, 2.5)	CH	9	C6, C9a	
9	125.9	7.62	br d (8.5)	CH		C5a, C7, C10	
9a	137.3			C			
10	39.2	3.73	br s	CH_2		C1, C5a, C9, C9a, C10a	10a-SMe
10a	71.8			C			
10a-SMe	13.2	2.27	S	CH_3		C10a	10

[a] Measured at 125 MHz. [b] Measured at 500 MHz.

EXPERIMENTAL SECTION

General Experimental Procedures. A Waters 1525 system with a Phenomenex Luna C18 (2) 5 μm column (4.6 × 150 mm) and a PDA detector was used for the HPLC analysis. Low-resolution MS data were measured using an Agilent Technologies VS/Agilent 1100 LC/MS system equipped with a Phenomenex Luna C18 (2) 5 μm column (4.6 × 150 mm, flow rate 0.7 mL/min). Lichroprep RP-18 (Merck, 40 μm) was used for the reversed-phase flash column chromatography. The HPLC separation and purification were performed using a Gilson 321 HPLC system with a Phenomenex Luna C18 (2) 10 μm column (21.2 × 250 mm) at a flow rate of 10 mL/min or with a Phenomenex Luna C18 (2) 10 μm column (10.0 × 250 mm) at a flow rate of 4 mL/min. All of the NMR spectra were obtained in methanol-d_4 (δ_H 3.32, δ_C 47.6) using a Varian UNITY Plus 500 MHz NMR system that was equipped with a cryogenic probe. The FT-IR spectra were recorded using a Bruker Tensor 27 spectrometer. The UV spectra were obtained using an Agilent 8453 UV/vis spectrophotometer. The CD spectra were measured using a JASCO J-810 spectropolarimeter. The optical rotations were measured using a P2010 JASCO polarimeter. The HRFABMS data were obtained using a JEOL/JMS-AX505WA instrument.

Strain Collection, Isolation, and Identification. The isolation and identification of the producing strains, *Sphingomonas* sp. KMK-001 and *Aspergillus fumigatus* KMC-901, were performed according to previously published methods.[3]

Cultivation and Extraction. The bacterial strain KMK-001 and the fungal strain KMC-901 were cultured in 8 L and 500 mL volumes of Czapek-Dox liquid culture medium (30 g saccharose, 3 g sodium nitrate, 1 g dipotassium phosphate, 0.5 g magnesium sulfate, 0.5 g potassium chloride, and 0.01 g ferrous sulfate in 1 L of distilled water) at 25 °C with shaking at 200 rpm, respectively. After 2 days, 250 μL of the KMC-901 culture broth (approximately 200 spores/mL) was inoculated into 500 mL of the bacterium KMK-001 culture broth (approximately 10^5 cfu/mL). After inoculation of fungus KMC-901, a time-course analysis was performed on the secondary metabolites once every two days. Each extract was analyzed using a Waters analytical HPLC with a Phenomenex Luna 5 μm C-18 (2) column (4.6 × 150 mm) and a gradient elution from 10% to 100% aqueous acetonitrile over 30 min. After 18 days, the cocultured broth was extracted with 16 L of ethyl acetate. The ethyl acetate extract was dried *in vacuo* to yield 1.3 g of crude extract.

Isolation and Purification. The ethyl acetate extract (1.3 g) was fractionated using Lichroprep RP-18 (40−63 μm, Merck, NJ, USA) flash column chromatography and a step gradient elution with H_2O and CH_3CN (20%, 40%, 60%, 80%, and 100% acetonitrile in H_2O) to yield six subfractions. The 60% acetonitrile fraction was dried *in vacuo* and fractionated using reversed-phase HPLC (Gilson 321; Phenomenex Luna 10 μm C18 (2) 250 × 10 mm column; 4 mL/min) and a gradient elution from 10% to 100% aqueous CH_3CN for 1 h. Glionitrin B (**1**, 3 mg, t_R = 15 min) was purified using normal-phase HPLC (Gilson 321; Phenomenex Luna 10 μm silica 250 × 10 mm column, 4 mL/min) with the elution of 60% DCM in EtOAc as solvent.

*Glionitrin B (**1**):* pale yellowish, amorphous gum; $[\alpha]_D^{25}$ −7.7 (*c* 0.10, MeOH); UV (MeCN) λ_{max} (log ε) 328 (3.06), 252 (3.93), 196 (4.14) nm; IR (film) ν_{max} 3447, 2924, 1674, 1603, 1526, 1480 cm^{-1}; ^{1}H and ^{13}C NMR spectra, see Table 1; HRFABMS $[M + H]^+$ *m*/*z* 384.0686 (calcd for $C_{15}H_{18}N_3O_5S_2$, 384.0688).

Reduction and Methylation of Glionitrin A (2). Iodomethane (500 μL) was added to a solution of glionitrin A (**2**, 5 mg) in pyridine (100 μL) at 0 °C, and the suspension was dissolved by adding methanol (200 μL). Sodium borohydride (10 mg) in methanol (200 μL) and iodomethane (200 μL) were added successively, and the resulting mixture was stirred at room temperature for 6 h. The final solution was evaporated *in vacuo* and partitioned between water and ethyl acetate. The ethyl acetate fraction was subjected to normal-phase HPLC separation (Gilson 321; Phenomenex Luna 10 μm silica 250 × 10 mm column, 4 mL/min) using the elution of 60% DCM in EtOAc as solvent to obtain the semisynthetic glionitrin B (3 mg).

Semisynthetic Glionitrin B. ^{1}H NMR (500 MHz, methanol-d_4) δ 8.83 (1H, d, *J* = 2.0 Hz, H-6), 8.14 (1H, br dd, *J* = 8.5, 2.0 Hz, H-8), 7.62 (1H, br d, *J* = 8.5 Hz, H-9), 4.39 (1H, d, *J* = 11.5 Hz, H-3a$_\alpha$), 3.98 (1H, d, *J* = 11.5 Hz, H-3a$_\beta$), 3.73 (2H, br s, H-10), 3.20 (3H, s, H-2), 2.36 (3H, s, SCH_3-3), 2.27 (3H, s, SCH_3-10a); ^{13}C NMR (125 MHz, methanol-d_4) δ 165.9 (C, C-1), 162.7 (C, C-4), 147.8 (C, C-7), 141.6 (C, C-5a), 137.0 (C, C-9a), 125.7 (CH, C-9), 120.9 (CH, C-8), 111.9 (CH, C-6), 72.1 (C, C-3), 71.5 (C, C-10a), 63.6 (CH_2, C-3a), 39.0 (CH_2, C-10), 27.9 (CH_3, 2-NCH_3), 13.0 (CH_3, 10a-SCH_3), 12.2 (CH_3, 3-SCH_3).

Cell Culture. The human prostate cancer cell line DU145 was purchased from the American Type Culture Collection (ATCC, VA, USA). The cells were grown in Eagle's minimum essential medium (Gibco BRL, MD, USA) that was supplemented with 10% fetal bovine

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serum (Gibco BRL), 100 units/mL penicillin, and 100 μg/mL streptomycin. Then, the cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Analysis of Cell Viability. The cells were treated with different concentrations of compounds **1** through **4** for 24 h, and the cell viability was measured using the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan), according to the manufacturer's recommendations. Paclitaxel (0.1 μM) was used as a positive control, which exhibited cell viability 18.9% of an untreated group in DU145 cancer cells.

Cell Invasion Assay. The cells were treated with the indicated concentrations of the compounds for 24 h. Next, the *in vitro* invasiveness was determined using a Cell Invasion Assay Kit (Chemicon, CA, USA), according to the manufacturer's protocol.

Total RNA Extraction and Reverse Transcriptase (RT)-PCR Analysis. After the treatment of the compounds for 18 h, the total RNA was isolated using an RNeasy Mini Kit (Qiagen, MD, USA) and reverse transcribed into cDNA using Superscript RNase H^- reverse transcriptase (Invitrogen, CA, USA), according to the manufacturer's recommendations. The subsequent PCR analysis was conducted with aliquots (100 ng) of the cDNA preparation using a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA). The PCR conditions were as follows: predenaturation at 94 °C for 5 min, which was followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min. The PCR products were visualized in 2% agarose gels. The primer sequences were as follows: MMP-2, 5′-ggC CCT gTC ACT CCT gAg AT-3′ (sense), 5′-ggC ATC CAg gTT ATC ggg gA-3′ (antisense); MMP-9, 5′-CAA CAT CAC CTA TTg gAT CC-3′ (sense), 5′-Cgg gTg TAg AgT CTC TCg CT-3′ (antisense); GAPDH (as an internal control for PCR), 5′-Cgg AgT CAA Cgg ATT Tgg TCg TAT-3′ (sense), 5′-AgC CTT CTC CAT ggT ggT gAA gAC-3′ (antisense).

■ ASSOCIATED CONTENT

Supporting Information. The spectroscopic data and biological activity results are available free of charge via the Internet at http://pubs.acs.org.

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