Glionitrin A, an Antibiotic−Antitumor Metabolite Derived from Competitive Interaction between Abandoned Mine Microbes

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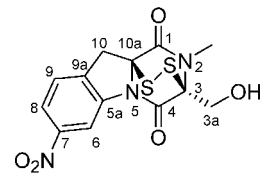
The nutrient conditions present in abandoned coal mine drainages create an extreme environment where defensive and offensive microbial interactions could be critical for survival and fitness. Coculture of a mine drainage-derived *Sphingomonas* bacterial strain, KMK-001, and a mine drainage-derived *Aspergillus fumigatus* fungal strain, KMC-901, resulted in isolation of a new diketopiperazine disulfide, glionitrin A (**1**). Compound **1** was not detected in monoculture broths of KMK-001 or KMC-901. The structure of **1**, a (3*S*,10a*S*) diketopiperazine disulfide containing a nitro aromatic ring, was based on analysis of MS, NMR, and circular dichroism spectra and confirmed by X-ray crystal data. Glionitrin A displayed significant antibiotic activity against a series of microbes including methicillin-resistant *Staphylococcus aureus*. An *in vitro* MTT cytotoxicity assay revealed that **1** had potent submicromolar cytotoxic activity against four human cancer cell lines: HCT-116, A549, AGS, and DU145. The results provide further evidence that microbial coculture can produce novel biologically relevant molecules.

Natural products have provided many novel chemical scaffolds that have been used in modern drug development.[1] However, as the rate of known compound reisolation from terrestrial plants and microorganisms has been increasing for more than a decade,[2] the need to develop more efficient methods for the discovery of new natural products has never been greater.

One method for accessing untapped natural product resources is to look for organisms in unusual environments. One such environment is found in acidic mine drainages. The ecological pressures of high acidity, enrichments of heavy metals, and other pollutants that this extreme environment contains may be a driving force for ecological competition leading to the production of new secondary metabolites by endemic microorganisms.[3,4] The competition that results from coculturing of microbes can lead the microbes to produce molecules they might not produce when left undisturbed. In order to investigate the chemical potential of microbes growing in this extreme environment, we designed a series of microbial coculturing experiments on the basis of previously reported mixed microbial culture studies.[5−8] One of the first documented examples of novel natural products arising from microbial coculturing was recently reported by the Fenical laboratory at the Scripps Institution of Oceanography. They described the coculture addition of a marine bacterium to a marine fungus, resulting in the production of a new antibiotic molecule, pestalone,[6] as well as two new antibacterial cyclic depsipeptides, emericellamides,[7] and four potent cytotoxic pimarane diterpenoids, libertellenones.[8]

During our coculture screening experiments with bacteria and fungi isolated from acidic mine drainage samples, we investigated the secondary metabolites produced from the coculture of the bacterial strain KMK-001 and the fungal strain KMC-901. These microbes were isolated from an acidic coal mine drainage that was highly contaminated by heavy metals (209 ppm Fe, 6.7 ppm Mn, 0.8 ppm Pb, 0.8 ppm Cu) and sulfuric acid. Addition of the fungal strain into the bacterial culture broth resulted in the production of a new secondary metabolite, glionitrin A (**1**), which showed potent *in vitro* inhibition of cancer cell proliferation as well as antimicrobial activities. The structure of **1** is closely related to those of gliotoxin (**2**) and dehydrogliotoxin (**3**), which were also isolated from the cultured *Aspergillus* fungus. One interesting difference is the presence of a nitro functionality at C-7. The circular dichroism spectrum of **1** is opposite in sign of those of **2** and **3**. This data, supported by X-ray crystallography data, confirmed that the absolute configurations of the chiral centers of **1** were opposite those of **3**.

A small number of nitro-aromatic compounds have been isolated from fungi[9] and bacteria,[10] and no nitro-aromatic metabolites have been reported from *Aspergillus* or *Sphingomonas* species. A few nitro-diketopiperazines, such as the verticillins[11] and thaxatomins,[12] are known to occur in nature. Here, we report the isolation and structure elucidation of **1** and some of its biological activities.



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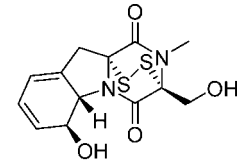

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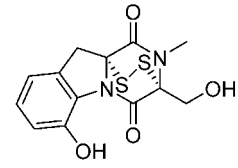

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Results and Discussion

Bacterial strain KMK-001 and fungal strain KMC-901 were isolated from an extremely contaminated acidic mine drainage collected from the Young-dong abandoned coal mine. The cocultured broth of bacterial strain KMK-001 and fungal strain KMC-901 was extracted with ethyl acetate. The ethyl acetate soluble fraction was subjected to a diversity of chromatographic purification steps to afford pure glionitrin A (**1**).

In order to confirm that **1** was truly a result of coculturing, we first investigated what molecules were produced by each microbial strain in pure culture. The *Sphingomonas* bacterial strain KMK-001 did not produce any notable secondary metabolites using our culture conditions. The pure culture of *Aspergillus fumigatus* fungal strain KMC-901 produced gliotoxin (**2**) and pseurotin A. Once the

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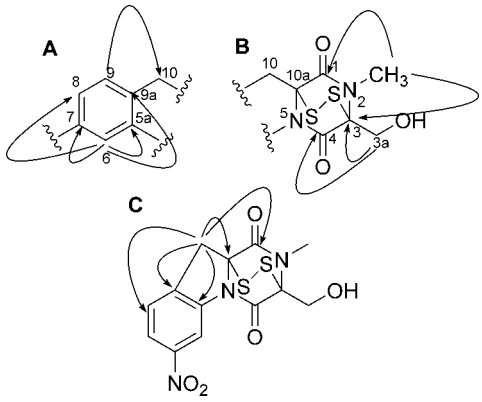

Figure 1. Key HMBC correlations of the partial structures of glionitrin A (**1**).

chemical components of the pure cultures had been analyzed, we then commenced coculture experiments by adding a small aliquot of fungal strain KMC-901 to a liquid culture of bacterial strain KMK-001. Coculture microbial growth and metabolite production was monitored daily by HPLC analysis. The HPLC analysis showed the production of a secondary metabolite that was not observed when the strains were grown separately in pure cultures. The metabolite was not observed until eight days of coculture, while **2** was observed on the third day and decreased after 10 days of coculture. A corresponding decline in bacterial density was observed with the production of **1**. NMR analysis of the new peak observed by HPLC indicated that it was a new secondary metabolite with the central core of a diketopiperazine ring. On the basis of its chemical relationship to gliotoxin, we named the new molecule glionitrin A (**1**).

Compound **1** was obtained as an optically active, pale yellow, amorphous powder having the molecular formula $C_{13}H_{11}N_3O_5S_2$, as determined by HRFABMS (obsd $[M + H]^+$ at *m/z* 354.0222, calcd, 354.0218) and ^{13}C NMR data. The ^1H NMR spectrum of **1** showed three aromatic proton signals (δ_H 8.75, 8.14, and 7.53), geminal benzylic signals (δ_H 4.40 and 3.44), hydroxymethylene signals (δ_H 4.52 and 4.34), a methyl group attached to nitrogen (δ_H 3.30), and a D_2O exchangeable doublet of doublets (δ_H 3.42). ^{13}C NMR data of **1** indicated two carbonyl carbons (δ_C 165.5 and 161.4), an *N*-methyl carbon (δ_C 27.8), two methylene carbons (δ_C 60.9 and 36.8), two quaternary carbons (δ_C 76.7 and 74.4), three aromatic methine carbons (δ_C 126.0, 121.7, and 111.4), and three aromatic quaternary carbons (δ_C 148.9, 139.2, and 135.3). Interpretation of HSQC data allowed all protons to be assigned to their respective carbons.

Partial structure A (A, Figure 1) was assigned using 2D NMR including $^1H-^1H$ COSY, HSQC, and HMBC spectrscopic data. The HMBC data showed connectivity of H-6 and H-8 through quaternary carbon C-7 (δ_C 148.9). The HMBC correlations between H-6, H-8, and the quaternary aromatic carbon C-9a (δ_C 135.3), and between H-9, C-5a (δ_C 139.2), and C-10 (δ_C 36.8) indicated the presence of a 1,3,4-trisubstituted phenyl group and allowed assignment of the three aromatic protons. The chemical shifts of H-6 (δ_H 8.75), H-8 (δ_H 8.14), and C-7 (δ_C 148.9) suggested the presence of a functionality with strong electron-withdrawing effects, such as a NO_2 group,[13] at C-7. The NO_2 functionality was supported by characteristic IR absorptions at 1527 and 1349 cm^{-1}.[14]

Substructure B (B, Figure 1) was assembled starting with the *N*-methyl resonance that showed HMBC correlations to carbons C-1 (δ_C 165.5) and C-3 (δ_C 76.7). The H_2-3a methylene signal (δ_H 4.34 and 4.52) showed HMBC correlations with C-3 (δ_C 76.7) and C-4 (δ_C 161.4). These data indicated that partial structure B was composed of two amide carbonyls (δ_C 165.5 and 161.4) and an *N*-methyl serine residue.

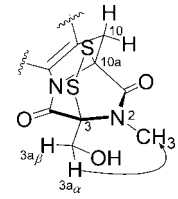

Figure 2. Key 1D NOE correlation of glionitrin A (**1**).

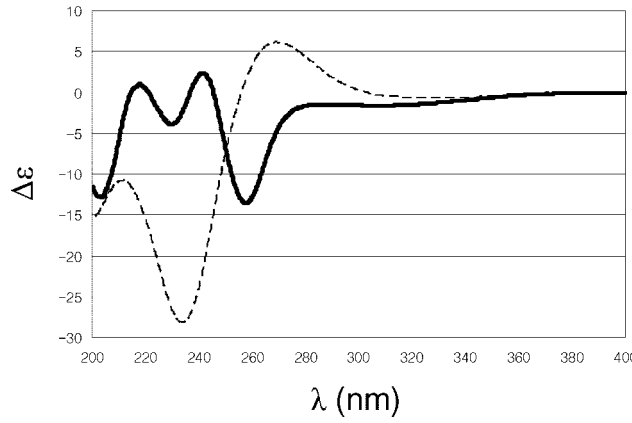

Figure 3. CD spectra of glionitrin A (**1**) (—) and gliotoxin (**2**) (---) in acetonitrile.

Partial structures A and B were connected on the basis of chemical shift analysis of the carbons adjacent to C-10 and the presence of an HMBC correlations from the H-10 endocyclic methylene signal to C-1, C-5a, C-9, C-9a, and C-10a (C, Figure 1). Finally, two quaternary carbons C-10a (δ_C 74.4) and C-3 (δ_C 76.7) were bonded by a disulfide group. This disulfide bond was assigned on the basis of the chemical shifts of C-10a and C-3,[15] together with HRFABMS data. The relative configuration of the 3,6-dithio-diketopiperazine moiety of **1** was identical to those of gliotoxin and dehydrogliotoxin based on a 1D selective ^1H NOE experiment (Figure 2). As expected, NOE correlations were observed only between the *N*-Me and the H-3a_α in the serine residue. These data indicated that glionitrin A (**1**) possessed the same boat diketopiperazine ring conformation created by the axial disulfide bond as in gliotoxin (**2**) and dehydrogliotoxin (**3**).

Assignment of the absolute configuration of **1** was initially determined by comparison of its CD spectrum to those of **2** and **3**, which have a 3*R*,10a*R* configuration at the disulfide bridge carbons. The CD spectra of the diketopiperazine disulfides, including **2**, are somewhat complex. However, characteristic absorbances at 340 nm due to the disulfide $n\rightarrow\sigma^*$ transition, 310 nm due to the $n_1\rightarrow\pi^*$ charge transfer, 270 nm due to the n_2, $n_3\rightarrow\pi^*$ charge transfer, and 240 nm due to overlapping of the peptide $n\rightarrow\pi^*$ transition and the disulfide n_2, $n_3\rightarrow\sigma^*$ transition are critical to the absolute configuration analysis.[16] The disulfide group of **2** has been shown to have a left-handed chirality, which showed a weak negative Cotton effect near 240 nm, a strong positive Cotton effect near 270 nm, and a weak negative Cotton effect near 340 nm. Surprisingly, **1** showed opposite signs to those of **2** and **3** at the characteristic CD absorbances (Figure 3).[17] Thus, the absolute configuration of the disulfide bridge carbons in **1** are likely *S,* as is seen in related molecules such as chaetocin, which has right-handed chirality.[18] The interpretation of the CD spectrum of **1** thus suggests an *S* configuration at the 3- and 10a-chiral carbons. X-ray crystallography was used to confirm the absolute configuration of the disulfide bond as well as the presence of an NO_2 funtionality in **1**. As shown in Figure 4, the absolute configuration was assigned by CD analysis and was confimed through X-ray crystallographic examination (see Experimental Section). Consequently, glionitrin A (**1**) was con-

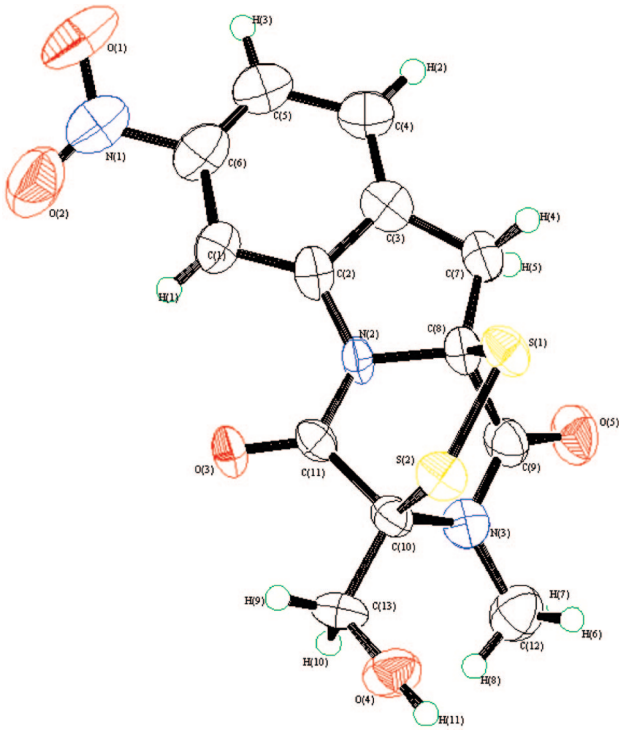

Figure 4. ORTEP drawing of the X-ray crystal structure of glionitrin A (**1**).

firmed to be a (3*S*,10a*S*) diketopiperazine disulfide containing an aromatic nitro group.

Throughout this work an equilibrium mixture of three main peaks was observed in the process of reversed-phase HPLC when an aqueous solution of **1** was exposed to ambient light in a capped clear vial. The LC/MS chromatogram of the mixture showed two additional peaks whose molecular ion peaks were observed at *m*/*z* 385 and 417, respectively. Polysulfurization of diketopiperazine disulfide analogues has been reported by Safe et al., who demonstrated that the polysulfide could be photochemically converted into an equilibrium mixture.[19] The two peaks derived from the photoconversion of **1** could not be fully purified in this study. However, the NMR data of the equalized mixture and the LC/MS trace suggested that these two peaks could be glionitrin-containing tri- and tetrasulfides.

Although we have strong evidence that glionitrin A (**1**) is produced as a result of coculturing, the question still remains as to its biological origin. We approached this question from several directions. Initially we considered four different mechanisms for the coculture production of **1**. One possibility is that once gliotoxin is produced by the fungus, it then undergoes a simple enzymatic transformation or postmodification by the bacterium.[20] Other options include the alteration of the fungal genetic expression of **1** by an unknown bacterial stimulus,[21] induction of novel production by external stressors, or a long-term competitive interaction between the bacterium and fungus.

The first experiment performed was to determine if glionitrins are made by bacterial enzymatic biotransformation or by postmodification of **2**, the major constituent of the pure culture broth of the fungal strain KMC-901. In one experiment we added the complete fungal organic extract to the bacterial culture broth, and in a second experiment we added pure **2**. With these additions, neither culture produced **1** at the end of the culture period, suggesting that bacterial enzymatic transformation was not the production mechanism.

The second hypothesis tested was whether an unknown bacterial stimulus was altering the genetic expression of the fungal metabolites by the fungus. This was tested through time course production experiments. When **1** was detected by HPLC, both the bacterium and the fungus were then isolated from the mixed culture broth. Pure cultures of each microbe were then grown independently using the same culture conditions. Analysis of the organic extracts from the resulting homogeneous cultures failed to show production of **1**, which had been detected in the coculture, indicating that this hypothesis was incorrect.

Table 1. Antimicrobial Bioassay Results for Glionitrin A (**1**)

	pathogen	MIC (μg/mL) **1**[a]	MIC (μg/mL) PC[b]
fungi	*Aspergillus fumigatus* HIC6094	12.5	1.56
	Trichophyton rubrum IFO9185	12.5	1.56
G(+) bacteria	*Micrococcus leuteus* IFC12708	0.78	0.78
	Bacillus subtilis ATCC6633	6.25	3.13
G(−) bacteria	*Proteus vulgaris* ATCC3851	3.13	1.56
	Salmonella typhimurium ATCC14028	3.13	12.50
MRSA	*Staphylococcus aureus* ATCC43300	0.78	12.50
	Staphylococcus aureus ATCC700787	0.78	12.50
	Staphylococcus aureus ATCC700788	0.78	25.00

[a] **1** = glionitrin A. [b] Positive control (PC) = amphotericin B for antifungal activities, ampicilin for antibacterial activities.

Finally, we investigated whether **1** could be produced solely by the fungus, but induced through external stressors. We explored this hypothesis by adding several different chemicals including phenol, nystatin, and H_2SO_4 into the pure fungal culture media. In a separate set of experiments we tested this hypothesis by adding the bacterial culture broth and cellular lysate, as well as extracts of the organic products from the bacterial culture, and the bacterial cell lysate into the fungal culture. None of these experiments resulted in the production of **1** by the fungal culture. On the basis of these results, we presume glionitrin A (**1**) derives from long-term competitive interactions between the microbes and involves an unknown mechanism.

Previous chemical studies of *Sphingomonas* species reported the presence of carotenoids,[22] polysaccharides,[23] sphingolipids,[24] and pentapeptides.[25] However no nitro compounds were reported from these bacteria, and they actually have the ability to degrade various xenobiotic substances including nitroaromatic compounds.[26] There were reports on several diketopiperazines,[27] a multimodular non-ribosomal peptide synthetase,[28] and a nitrate-assimilation gene cluster from *Aspergillus fumigatus*,[29] although no nitroaromatic metabolites have been isolated from this fungus. Glionitrin A (**1**) may have originated from the fungus through interactions with *Sphingomonas* strain KMK-001.

When **1** was examined for *in vitro* cytotoxicity against six human cancer cell lines, it showed submicromolar inhibition of cell proliferation in the HCT-116, A549, AGS, and DU145 cell lines (IC_{50} = 0.82, 0.55, 0.45, and 0.24 μM, respectively) and relatively weak inhibition of the cell proliferation of the MCF-7 and HepG2 cell lines (IC_{50} = 2.0 and 2.3 μM, respectively).

Antimicrobial activity of **1** was also evaluated against several microbial pathogens. As shown Table 1, **1** showed weak antimicrobial activity against two fungal strains, but showed promising antibacterial activity toward three strains of methicillin-resistant *Staphylococcus aureus* with significant MIC values of 0.78 μg/mL. This study is an example of how the microbial competition that occurs in coculture experiments can result in the production of novel chemical structures and potential drug candidates.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a P2010 JASCO polarimeter. UV/vis and FT-IR spectra were obtained on an Agilent 8453 UV/vis spectrophotometer and a Bruker Tensor 27 spectrometer, respectively. CD spectra were measured on a JASCO J-810 spectropolarimeter. NMR spectra were obtained in $CDCl_3$ on a Varian UNITY Plus 500 MHz spectrometer. NMR chemical shifts were referenced to the residual solvent peaks (δ_H 7.27 and δ_C 77.3 for $CDCl_3$). Low-resolution ESIMS were measured on an Agilent Technologies VS/Agilent 1100 system. HRFABMS data were obtained on

a JEOL/JMS-AX505WA instrument. X-ray diffraction intensity data were acquired with a Rigaku R-Axis Rapid-S single-crystal X-ray diffractometer with graphite-monochromated Mo Kα radiation (λ = 0.71075 Å). Lichroprep RP-18 (Merck, 40−63 μm) was used for column chromatography. Semipreparative HPLC separations were performed using a Gilson 321 HPLC system with a Phenomenex Luna C18(2) 10 μm column (10 × 250 mm) and a Luna silica 10 μm column (10 × 250 mm) at a flow rate of 4 mL/min. HPLC-MSD was performed with an Agilent 1100 LC-MS system using a Agilent Eclipse XDB-C18 5 μm column (4.6 × 150 mm). A Waters 1525 HPLC-PDA system with an Agilent Eclipse XDB-C18 5 μm column (4.6 × 150 mm) or Phenomenex Luna 5u C-18(2) analytical column (4.6 × 100 mm) was used for the analysis of extracts and fractions.

Sample Collection, Isolation, and Genetic Identification. Bacterial strain KMK-001 and fungal strain KMC-901 were isolated from an extremely contaminated acid mine drainage (including 209 ppm Fe, 6.7 ppm Mn, 0.8 ppm Pb, 0.8 ppm Cu, pH 3.0) collected from a horizontal pit, situated at an elevation of 750 m at the Young-dong abandoned coal mine located at Gangneung, South Korea. They were cultured on Czapek-Dox agar medium at 25 °C (30 g saccharose, 3 g sodium nitrate, 1 g dipotassium phosphate, 0.5 g magnesium sulfate, 0.5 g potassium chloride, 0.01 g ferrous sulfate, and 15 g agar in 1 L of distilled H_2O). The KMK-001 was identified as a *Sphingomonas* sp. Gram-negative bacterium on the basis of 98.0% 16S rDNA sequence similarity with *Sphingomonas* sp. A1XXyl1-5. The fungal strain KMC901 was identified as *Aspergillus fumigatus* on the basis of 100% 5.8S rDNA sequence similarity with *A. fumigatus*. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KMK-001 is EU219973. The cultured bacterial strain KMK-001 and the cultured fungal strain KMC-901 were deposited with Korean Culture Center of Microorganisms (KCCM 10888P and KCCM 10889P, respectively).

Isolation of Ribosomal DNA, Gene Amplification, and Sequencing. Isolation of the chromosomal DNA of bacterial strain KMK-001 and fungal strain KMC-901 was performed using a G-spin TM Genomic DNA extraction kit. From the genomic DNA, the 16S rDNA gene was amplified by polymerase chain reaction (PCR) using universal primers 27f and 1492r corresponding to positions 27 in the forward direction and 1492 in the reverse direction of the *E. coli* 16S rDNA sequence.[30] In the case of fungal strain KMC-901, ITS1, ITS2, and the inverting 5.8S coding rDNA were amplified by PCR using the primers ITS1 and ITS4 as previously described.[31]

The PCR reaction agents were prepared by mixing 1.5 μL of template DNA, 5 μL of 10× Taq buffer, 1 μL of 10 mM mixed dATP, dTTP, dGTP, and dCTP, 2 μL of sense-27F primer (10 pmol/μL), 2 μL of antisense-1492R primer (10 pmol/μL), 10 pmol/μL each of the primers ITS1 and ITS4 in the case of fungal strain KMC-901, and 5 U of *Taq* DNA polymerase in a final volume of 50 μL. The thermal conditions were as follows: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), extension (1 min and 30 s at 72 °C), and final extension at 72 °C for 7 min. The 16S and 5.8S rDNA sequence determination was performed using a Perkin-Elmer model ABI 3730XL capillary DNA sequencer.

Cultivation and Extraction. Bacterial strain KMK-001 was cultured in 1 L Erlenmeyer flasks (16 × 500 mL, total 8 L) each containing 500 mL of Czapek-Dox broth (30 g saccharose, 3 g sodium nitrate, 1 g dipotassium phosphate, 0.5 g magnesium sulfate, 0.5 g potassium chloride, 0.01 g, ferrous sulfate in 1 L of distilled H_2O) at 25 °C with shaking at 200 rpm. The fungus was cultivated in a 1 L Erlenmeyer flask (500 mL of medium) in the same manner. After 2 days, a small volume of the culture broth of fungus KMC-901 was inoculated into each flask containing the culture broth of bacterium KMK-001 (250 μL per 500 mL). After inoculation of fungus KMC-901, time-course analysis was performed on the secondary metabolites once every two days. After 15 days of coculture the 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 of Glionitrin A (1). The crude extract was fractionated using Lichroprep 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 give six subfractions.

The 60% acetonitrile fraction was dried *in vacuo* and fractionated by reversed-phase HPLC using gradient elution from 10% to 100% aqueous CH_3CN. Glionitrin A (**1**, 20 mg) was purified by normal-phase HPLC (Phenomenex Luna 10 μm silica 250 × 10 mm column) eluting with 90% DCM in EtOAC. Glionitrin A (**1**) (5.0 mg) was crystallized in a glass insert with 500 μL of acetonitrile−H_2O (6:4). The cap of the vial was punctured with a needle, and the solution was heated for 1 min at 65 °C and was then kept at 4 °C. The resulting solution yielded numerous small, clear pale yellowish crystals.

Glionitrin A (1): pale yellow plates; mp 181−182 °C; $[\alpha]^{25}_D$ −254 (*c* 0.1, $CHCl_3$); UV (CH_3CN) λ_{max} (log ϵ) 327 (3.34), 251 (4.09), 194 (4.23) nm; IR (film) ν_{max} 3474, 2925, 1695, 1606, 1527, 1349 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 8.75 (1H, d, J = 2.5 Hz, H-6), 8.14 (1H, br dd, J = 8.5, 2.5 Hz, H-8), 7.53 (1H, br d, J = 8.5 Hz, H-9), 4.52 (1H, dd, J = 12.5, 6.0 Hz, H_2-3a$_\beta$), 4.40 (1H, br dd, J = 19.0, 1.0, H_2-10$_\beta$), 4.34 (1H, dd, J = 12.5, 10.0 Hz, H_2-3a$_\alpha$), 3.44 (1H, d, J = 19.0 Hz, H_2-10$_\alpha$), 3.42 (1H, dd, J = 10.0, 6.0 Hz, 3a-OH), 3.30 (3H, s, 2-NCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 165.5 (C, C-1), 161.4 (C, C-4), 148.9 (C, C-7), 139.2 (C, C-5a), 135.3 (C, C-9a), 126.0 (CH, C-9), 121.7 (CH, C-8), 111.4 (CH, C-6), 76.7 (C, C-3), 74.4 (C, C-10a), 60.9 (CH_2, C-3a), 36.8 (CH_2, C-10), 27.8 (CH_3, 2-NCH_3); HRFABMS $[M + H]^+$ *m/z* 354.0222 (calcd for $C_{13}H_{12}N_3O_5S_2$, 354.0218).

X-ray Study of Glionitrin A (1). A yellow platelet crystal of **1** having approximate dimensions of 0.40 × 0.10 × 0.05 mm was mounted on a glass fiber. All measurements were made on a Rigaku RAXIS RAPID imaging plate area detector with graphite-monochromated Mo Kα radiation. Indexing was performed from 2 oscillations that were exposed for 90 s. The crystal-to-detector distance was 127.40 mm. The data were collected at a temperature of 23 ± 1 °C to a maximum 2θ value of 54.9°. A total of 44 oscillation images were collected. A sweep of data was done using ω scans from 130.0° to 190.0° in 5.0° steps, at χ = 45.0° and ϕ = 0.0°. The exposure rate was 60.0 [s/deg]. A second sweep was performed using ω scans from 0.0° to 160.0° in 5.0° steps, at χ = 45.0° and ϕ = 180.0°. The exposure rate was 60.0 [s/deg]. The crystal-to-detector distance was 127.40 mm. Readout was performed in the 0.100 mm pixel mode. Of the 7443 reflections that were collected, 7428 were unique (R_{int} = 0.042); equivalent reflections were merged. The linear absorption coefficient, μ, for Mo Kα radiation is 3.7 cm^{-1}. An empirical absorption correction was applied that resulted in transmission factors ranging from 0.79 to 1.00. The data were corrected for Lorentz and polarization effects. The structure was solved by direct methods and expanded using Fourier techniques. The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were refined using the riding model. The final cycle of full-matrix least-squares refinement on F^2 was based on 3450 observed reflections and 209 variable parameters. CCDC 699396 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request /cif.

Studies Relating to the Possibile Mechanism of Glionitrin A (1) Production. *Sphingomonas* bacterial strain KMK-001 was cultured in two 1 L Erlenmeyer flasks containing 500 mL of Czapek-Dox broth at 25 °C with shaking at 200 rpm. After three days, gliotoxin (0.01% w/v, 5 mg/flask) and the ethyl acetate extract of the strain KMC-901 culture broth (0.1% w/v, 50 mg/flask), in separate experiments, were inoculated into each bacterial culture broth. Each sample was analyzed once every two days for 15 days. None of these experiments resulted in the production of glionitrin A (**1**).

When **1** was detected from the coculture broth, 50 μL of the culture broth was inoculated onto the surface of an Czapek-Dox agar plate and spread with a sterilized glass rod, and the plate was incubated for 7 days at room temperature. Each colony of the bacterium and the fungus was then isolated from the agar plate. Pure cultures of each microbe were then grown independently in 500 mL of Czapek-Dox culture broth at 25 °C with shaking at 200 rpm. Each culture broth was analyzed once every two days for 15 days. None of these experiments resulted in the production of **1**.

The fungal strain KMC-901 was cultured in four 1 L Erlenmeyer flasks containing 500 mL of Czapek-Dox broth. After three days, phenol (25 μL), nystatin (50 μg/mL), the cell lysate (10 mL) of the bacterial strain KMK-001 prepared by ultrasonication of the bacterial cells, and the ethyl acetate extract (100 mg) of bacterial culture broth, in separate experiments, were inoculated into each fungal culture flask. The fungal strain KMC-901 was cultured in the acidified Czpek-Dox broth (pH 3, 500 mL) with sulfuric acid. All cultures were allowed to proceed for 18 days. Each culture broth was analyzed once every two days. None of these experiments resulted in the production of **1**.

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Supporting Information Available: Spectroscopic data are available free of charge via the Internet at http://pubs.acs.org.

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