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Research Note

Antifungal Substances from *Streptomyces* sp. A3265 Antagonistic to Plant Pathogenic Fungi

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Abstract In a previous study, we identified a *Streptomyces* sp., A3265, as exhibiting potent antifungal activity against various plant pathogenic fungi, including *Botrytis cinerea*, *Colletotrichum gloeosporioides*, and *Rhizoctonia solani*. This strain also exhibited a biocontrolling effect against ginseng root rot and damping-off disease, common diseases of ginseng and other crops. In this study, we isolated two antifungal substances responsible for this biocontrolling effect via Diaion HP-20 and Sephadex LH-20 column chromatography, medium pressure liquid chromatography, and high-performance liquid chromatography. These compounds were identified as guanidylfungin A and methyl guanidylfungin A by spectroscopic methods. These compounds exhibited potent antimicrobial activity against various plant pathogenic fungi as well as against bacteria.

Keywords Biocontrol agent, Damping-off, Guanidylfungin, *Streptomyces* sp. A3265

Ginseng (*Panax ginseng*) is a highly valued herb in various regions of China, Korea, North America, and other countries. It has been used as a general tonic in traditional oriental medicine. Ginseng powder from roots is a component of many value-added products, such as medicine, dietary supplements, drinks, soups, candies, and jellies [1]. Hence, ginseng is considered to be the most widely taken herbal medicine in the world. In recent studies, ginseng has been proven to possess various biological and pharmacological properties, such as antineoplastic, immunomodulatory, antibiotic, anti-inflammatory, anticancer, antioxidant, antiemetic, and antiproliferative properties, as well as other health benefits [2-6]. These biological and pharmacological properties are strongly related to the presence of phytochemicals such as saponins, alkaloids, polysaccharides, polyacetylenes, free amino acids, polyphenolics, and volatile compounds [7-9].

Ginseng yield is limited by many factors, including damping-off and root rot in fields as well as during storage prior to consumption. Damping-off can be controlled by using fungicides or biocontrol agents. There are many fungicides available to control damping-off caused by *Rhizoctonia solani* such as iprodione, benomyl, fentin hydroxide, benodianil, difenoconazole, and pencycuron [10-13]. Root rot caused by *Botrytis cinerea* accounts for the majority of postharvest loss in ginseng. Although much research has been conducted on the action of the disease during plant growth, little information is available on the control of postharvest root rot. Control of *B. cinerea* is normally carried out by the application of fungicides. However, the growing demand of consumers worldwide for a reduction in the use of fungicides, as well as the appearance of pathogens resistant to chemical compounds, has emphasized the need to find alternative methods for control of these ginseng diseases.

Biological control is an efficient and environmentally friendly way to prevent ginseng damping-off and root rots diseases. In recent years, actinobacteria has been validated as a potent biocontrol agent, because it can produce antifungal substances as secondary metabolites. There are several antifungal compounds have been found to have potent antifungal activity that controls plant fungal disease, such as a manumycin-type antibiotic [14], rhamnolipid [15], phenazine-1-carboxylic acid [16], aerugine [17], thibutacin [18], and staurosporine [19].

In a previous study, we identified a *Streptomyces* sp. A3265 exhibiting potent antifungal activity against various

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plant pathogenic fungi. In this study, we isolated two antifungal substances through Diaion HP-20 and Sephadex LH-20 column chromatography, medium pressure liquid chromatography, and high-performance liquid chromatography and characterized their structures by spectroscopic methods, mainly mass and nuclear magnetic resonance spectra.

Cultivation. *Streptomyces* sp. A3265 was maintained on a modified Bennett's agar plate at 27°C for 5 days and then inoculated into two 1-L Erlenmeyer flasks containing 200 mL of seed medium, consisting of glucose 20 g, soluble starch 10 g, meat extract 1 g, yeast extract 4 g, sodium chloride 2 g, potassium phosphate dibasic anhydrous 0.05 g, and soybean flour 25 g in 1 L water (pH 7.0 before sterilization). After incubation at 27°C for two days on a rotary shaker at 120 rpm/min, aliquots of this seed culture (2 mL) were transferred to fifteen 1-L Erlenmeyer flasks containing 200 mL each of the same medium as for the seed culture. The fermentation was carried out at 27°C for five days on a rotary shaker at 120 rpm/min.

Isolation of antifungal substances. After 5 days of fermentation, the culture broth was harvested and centrifuged at 6,000 rpm for 20 min to separate supernatant from precipitate. The precipitate was extracted with acetone for 24 hr. Antifungal activity against *Rhizoctonia solani* and *Botrytis cinerea* was evident in both the supernatant and the acetone extract of the precipitate. The acetone extract of the precipitate was evaporated *in vacuo*, dissolved in water, and combined with the supernatant. The combined broth was separated by Diaion HP-20 column chromatography eluting with methanol : water (0 : 100, 30 : 70, 50 : 50, 70 : 30, 100 : 0, v/v, stepwise). The active fraction (70% aqueous methanol eluate) was separated by Sephadex LH-20 (Phamarcia, Uppsala, Sweden) column chromatography eluting with 70% aqueous methanol, followed by preparative medium pressure liquid chromatography eluting with a gradient of increasing methanol concentration (40% to 100% methanol) in water. Fractions showing antifungal activity were combined, concentrated, and then dissolved in 30% aqueous acetonitrile and kept in a refrigerator overnight. The precipitate was collected and recrystallized in 30% aqueous acetonitrile to yield compound A3265-2. All of the supernatant from the crystallization was collected, evaporated, and finally separated by preparative high-performance liquid chromatography using 33% aqueous acetonitrile, followed by preparative high-performance liquid chromatography with C_{18} (RP-18; Cosmosil, Kyoto, Japan) column using 58% aqueous methanol containing 0.04% trifluoroacetic acid, to yield compound A3265-1 (Fig. 1).

Structure determination of antifungal substances. The chemical structures of compounds A3265-1 and A3265-2, which demonstrated antifungal activity, were determined by spectrocopic methods, as shown in Fig. 2. Electrospray ionization (ESI) mass measurements in positive

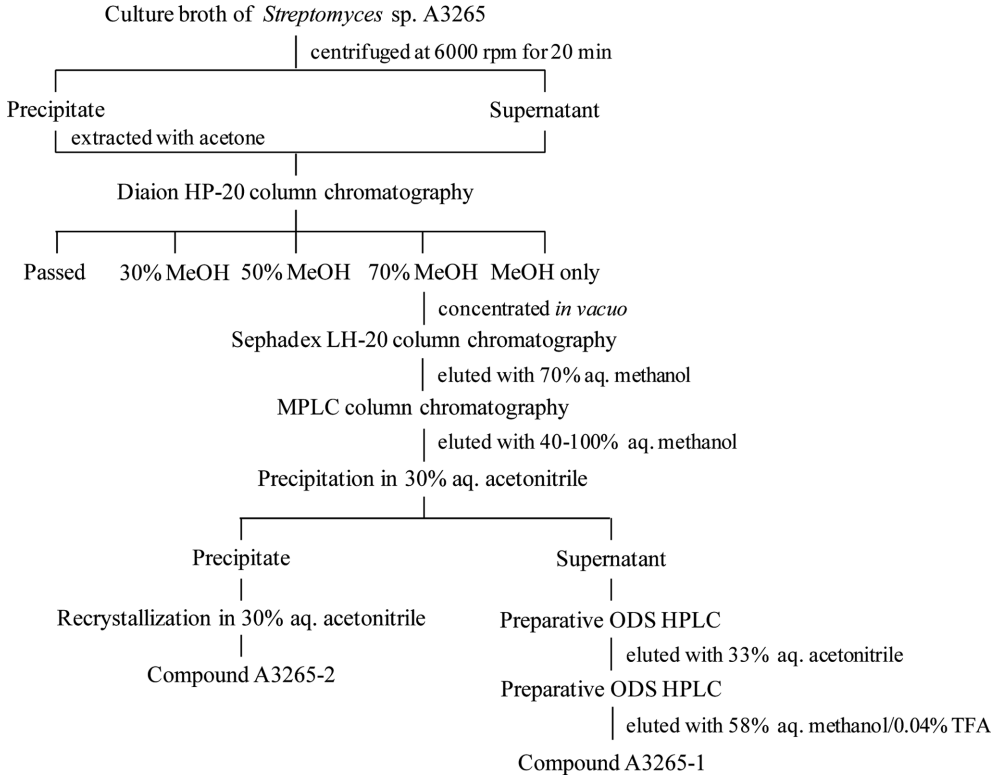
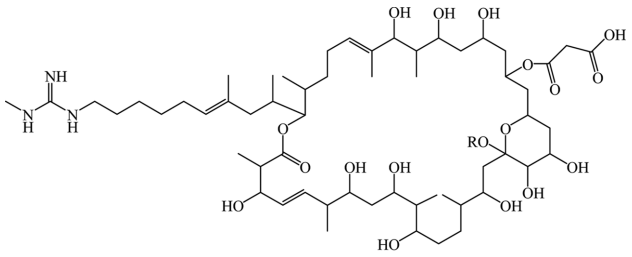


Fig. 1. Purification flow chart of antifungal compounds from culture broth of *Streptomyces* sp. A3265. MPLC, medium pressure liquid chromatography; HPLC, high performance liquid chromatography.



Compound A3265-1 (= guanidylfungin A) R = H
Compound A3265-2 (= methyl guanidylfungin A) R = CH_3

Fig. 2. Chemical structures of compounds A3265-1 and A3265-2.

mode revealed quasi-molecular ion peaks at *m/z* 1,130.4 $[M+H]^+$ and 1,152.3 $[M+Na]^+$ for compound A3265-1 and *m/z* 1,144.4 $[M+H]^+$ and 1,166.3 $[M+Na]^+$ for compound A3265-2. The ESI-mass measurements established their molecular weights to be 1,129 and 1,143, respectively (Fig. 3). The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of compound A3265-1 exhibited signals ascribable to four olefinic methine groups at δ 5.72, 5.44, 5.33, and 5.32, numerous oxygenated methine groups at δ 3.5-4.2, one *N*-methyl group at δ 2.82, and nine methyl groups at δ 0.5-1.5 (Fig. 4). In the carbon nuclear magnetic resonance (^{13}C-NMR) spectrum, 58 carbons, including three carbonyl carbons at δ 177.0, 170.5, and 169.1; one guanidyl carbon at δ 158.3; six olefinic carbons at δ 120-140; one hemiketal carbon at δ 102.9; oxygenated carbons at δ 60-85; methylene and methine carbons at δ 20-60; and nine methyl carbons at δ 10-18 were evident (Fig. 5). These spectral data were in good agreement with those of guanidylfungin A. The structure of A3265-1 was finally confirmed by 2-dimensional NMR measurements, including ^{1}H-^{1}H correlation spectroscopy (^{1}H-^{1}H COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple-bond coherence (HMBC) spectra, which were consistent with the structure of guanidylfungin A. Therefore, the structure of compound A3265-1 was determined to be guanidylfungin A [20, 21].

The ^{1}H-NMR spectrum of compound A3265-2 was very similar to that of compound A3265-1, except for the presence of one methoxyl group at δ 3.98 that was easily exchanged by solvent (-OCD_3). In the ^{13}C-NMR spectrum of compound A3265-2, 59 carbons were observed. The ^{13}C-NMR spectrum of A3265-2 was very similar to that of compound A3265-1, except for the presence of one methoxyl carbon at δ 54.9 that was easily exchanged by solvent (-OCD_3). The mass and NMR spectral data implied that compound A3265-2 was methyl guanidylfungin A. Two-dimensional NMR spectra of A3265-1, including ^{1}H-^{1}H COSY, HMQC, and HMBC, were consistent with methyl guanidylfungin A. Therefore, compound A3265-2 was identified to be methyl guanidylfungin A [20, 21].

Antimicrobial activity. The antimicrobial activities of compounds A3265-1 and A3265-2 isolated from *Streptomyces*

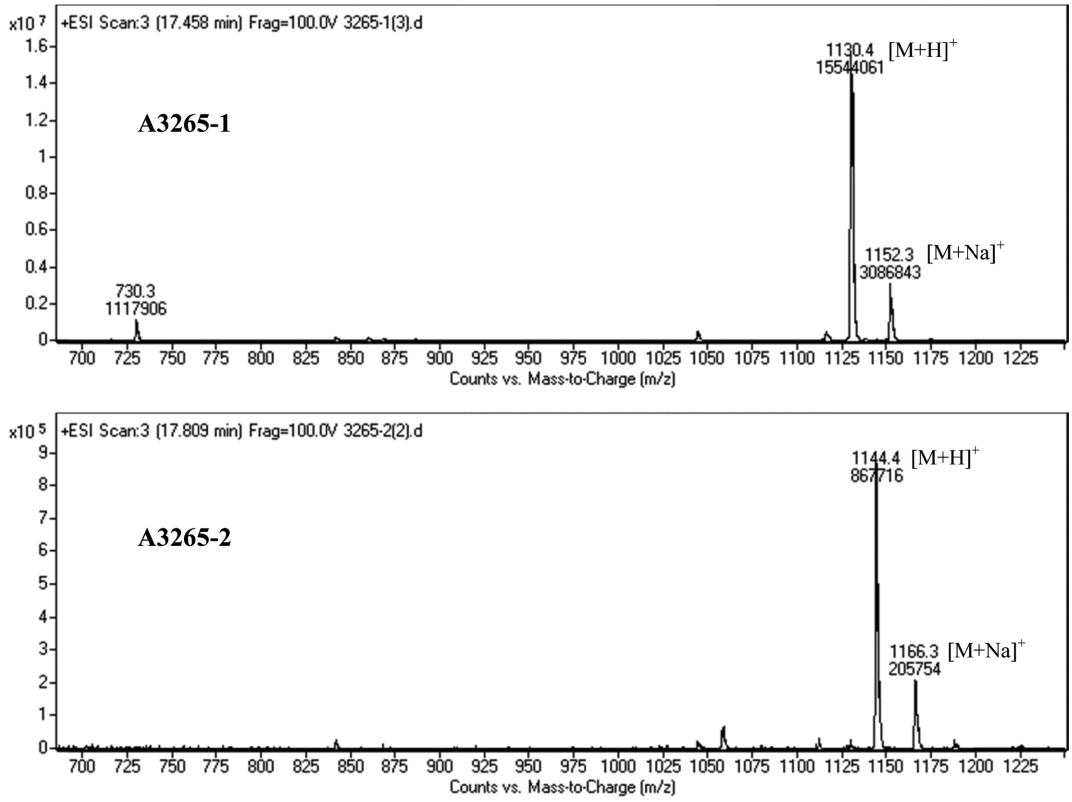


Fig. 3. Electrospray ionisation mass spectra of compounds A3265-1 and A3265-2 in positive mode.

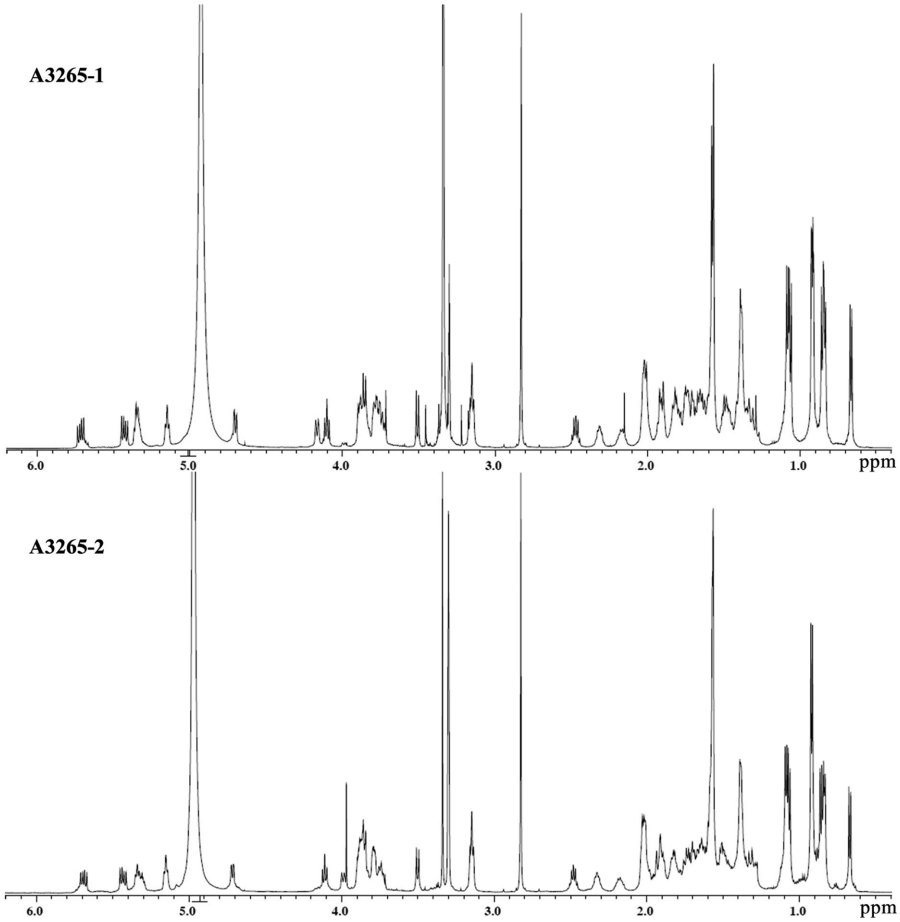


Fig. 4. [1]H-NMR spectra of compounds A3265-1 and A3265-2 in CD_3OD. [1]H-NMR, proton nuclear magnetic resonance.

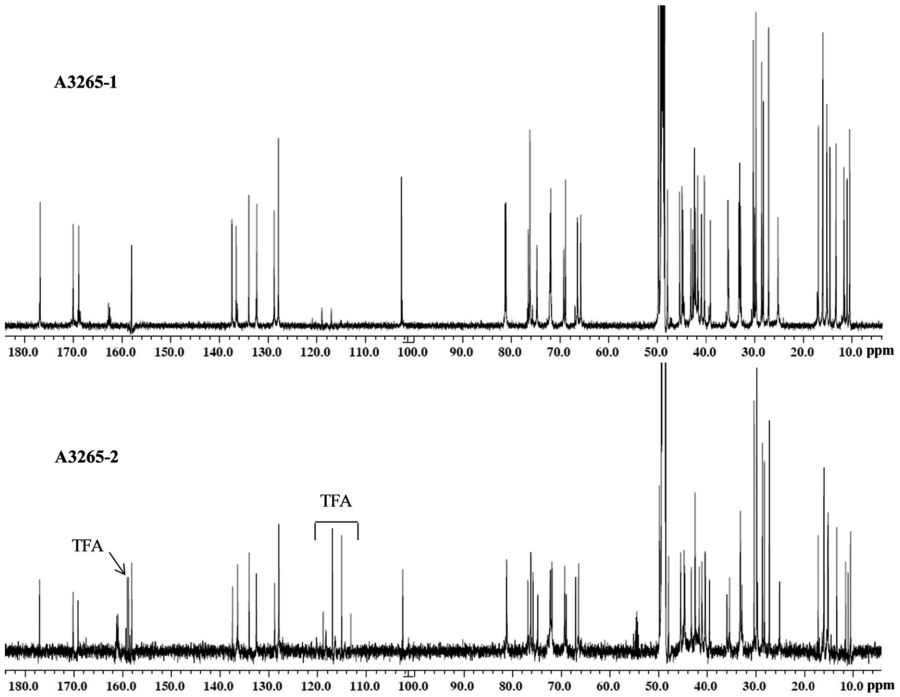


Fig. 5. [13]C-NMR spectra of compounds A3265-1 and A3265-2 in CD_3OD. [13]C-NMR, carbon nuclear magnetic resonance; TFA, trifluoroacetic acid.

Table 1. Antimicrobial activity of compounds A3265-1 and A3265-2

Microorganisms	Minimal inhibitory concentration (μg/mL)	
	Compound A3265-1	Compound A3265-2
Botrytis cinerea	20.0	2.5
Fusarium oxysporum	5.0	2.5
Alternaria panax	10.0	2.5
Colletotrichum gloeosporioides	20.0	10.0
Rhizoctonia solani	10.0	10.0
Magnaporthe grisea	20.0	10.0
Staphylococcus aureus	10.0	5.0
Bacillus subtilis	20.0	5.0
Escherichia coli	> 50.0	> 50.0

sp. A3265 were estimated using various plant pathogenic fungi, including *Alternaria panax*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, and *Magnaporthe grisea*, and bacteria including *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*. The results demonstrated that compounds A3265-1 and A3265-2 exert potent antifungal activity against plant pathogenic fungi (Table 1). Compound A3265-1 exhibited potent activity against *F. oxysporum* and *R. solani*, with minimal inhibitory concentrations (MICs) of 5.0 and 10 μg, respectively. Compound A3265-2 showed significant antifungal activity against *F. oxysporum* and *A. panax*, with a MIC value of 2.5 μg. Compound A3265-2 exhibited higher antifungal activity than did compound A3265-1. These compounds also exhibited antibacterial activity against Gram-positive bacteria, with MICs in the range of 10~20 μg. These results demonstrate that strain A3265 produces two antibiotics of broad spectrum with antifungal activity against various plant pathogenic fungi, as well as antibacterial activity.

Conclusion. The use of a microorganism to prevent plant disease offers an attractive alternative or supplement to synthetic fungicides for the management of plant disease without the negative effects of chemical control mechanisms. During screening for microorganisms with the potential to be used as microbial fungicides, *Streptomyces* sp. A3265 was identified as having potent antifungal activity against various plant pathogenic fungi, including *B. cinerea*, *C. gloeosporioides*, and *R. solani*. In *in vivo* testing, A3265 exhibited a biocontrolling effect against ginseng root rot and damping-off diseases of seeds and seedlings (data not shown, manuscript in preparation). In this study, we identified and studied the antifungal substances responsible for this biocontrolling effect. Two antifungal substances were purified from culture broth of *Streptomyces* sp. A3265 through Diaion HP-20 and Sephadex LH-20 column chromatography, medium pressure liquid chromatography, and high-performance liquid chromatography. These compounds were identified as guanidylfungin A and methyl guanidylfungin A via spectroscopic methods. These compounds exhibited potent antimicrobial activity against various plant pathogenic fungi as well as against bacteria. These results suggest that *Streptomyces* sp. A3265 has the potential to be a biocontrol agent of broad spectrum, effective against diverse plant diseases.

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