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Antifungal Activities of Metabolites Produced by a Termite-Associated *Streptomyces canus* BYB02

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ABSTRACT: Two main antifungal metabolites resistomycin and tetracenomycin D were isolated and purified from a termite-associated *Streptomyces canus* BYB02 by column chromatography. The structures of isolated compounds were determined on the basis of extensive spectroscopic analysis. Resistomycin possessed strong activities against mycelial growth of *Valsa mali* (IC_{50} = 1.1 μg/mL) and *Magnaporthe grisea* (IC_{50} = 3.8 μg/mL), which were comparable to those of referenced cycloheximide, with IC_{50} values of 2.3 and 0.3 μg/mL, respectively. A further spore germination test showed that resistomycin exhibited potent reduction in spore germination for *M. grisea*, with an IC_{50} value of 5.55 μg/mL. Finally, the *in vivo* antifungal activity experiment showed that resistomycin possessed significant preventive efficacy against rice blast, which was more potent than that of referenced carbendazim, with control efficacies of 66.8 and 58.7%, respectively. The present results suggest that resistomycin has potential to be used as a fungicide.

KEYWORDS: *Streptomyces canus, resistomycin, tetracenomycin D, antifungal activity*

INTRODUCTION

Although microbial antibiotic products from soil origin have been widely explored, the metabolites of those occupying some special ecological niches have not been fully investigated. The ability of insects to live in unique niche habitats is often facilitated by association with their microbial symbionts.[1,2] The class Insecta had 900 000 known and 2−30 million predicted species,[3] and it has been estimated that at least 15−20% of all insects live in symbiotic relationships with microbes.[4] The large insect species fostered the enormous symbiotic microbial communities, which were sources of novel antibiotic metabolites.[5,6] However, there have been only a few papers dealing with the bioactive compounds of the insect symbionts.[7,8] In our continuous identification of bioactive metabolites from the insect-derived microbiota,[9−11] we found that the ethyl acetate extract from the culture filtrates of the actinomycetic BYB02, isolated from *Odontotermes formosanus*, exhibited potent antifungal activities against several plant pathogens. Subsequent activity-monitored fractionation of the crude extract resulted in the isolation and characterization of two main bioactive metabolites named resistomycin and tetracenomycin D. Details of the isolation, structural elucidation, and bioactivity of the two metabolites were reported here.

MATERIALS AND METHODS

Isolation and Identification of Strain BYB02. According to the methods detailed elsewhere with some modifications,[12] the strain BYB02 was isolated from *O. formosanus* collected in August 2010 from the campus of Zhejiang Normal University, China. Briefly, the termites were surface-sterilized in 70% ethanol, followed by rinsing in sterile water, and then dried on the sterile filter paper. A total of 20 termites were placed in a mortar filled with 500 μL of NaCl solution (0.9%, w/v) and were crushed mechanically for 2 min using a hand-held glass homogenizer. The triturations were diluted in a 10-fold dilution series, and aliquots of 100 μL from each dilution were spread onto Gause's agar medium (consisting of 20 g of soluble starch, 1 g of KNO_3, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4{\cdot}0.7H_2O$, 0.5 g of NaCl, 0.01 g of $FeSO_4$, and 20 g of agar in 1 L of distilled water). The media were supplemented with 0.1 g/L cycloheximide to suppress the fungal growth. They were incubated aerobically for 96 h at 28 °C. The pure actinomycetic colony from the appropriate dilution was streaked on new Gause's medium. The isolated strain was preserved on Gause's medium slants at 4 °C until used. The title strain was identified by comparing the morphological character and 16S rRNA sequence to those of standard record.

Microbial Fermentation. The fresh mycelium grown on Gause's agar medium at 28 °C for 4 days was inoculated into a 500 mL Erlenmeyer flask containing 100 mL of Gause's liquid medium. After 2 days of incubation at 28 °C on rotary shakers at 150 rpm, 25 mL of cultural liquid was transferred as a seed into each 500 mL Erlenmeyer flask containing 100 mL of Gause's liquid medium. Cultivation was kept at 28 °C with an agitation of 180 rpm for 10 days. The procedure was repeated until sufficient biomass was accumulated.

Isolation and Characterization of the Bioactive Metabolites. The filtrate of the cultural broth (10 L) was extracted with ethyl acetate (4 × 10 L). Evaporation of menstruum *in vacuo* gave a yellow oily residue (5.4 g), which was subjected to chromatography over a silica-gel column eluting with CH_2Cl_2/MeOH mixtures of a stably growing polarity (100:0, 100:1, 100:2, and 100:4, v/v) to afford four parts (F1, 3.7 g; F2, 0.5 g; F3, 0.5 g; and F4, 0.3 g). The bioassay results showed that F2 (100:1 CH_2Cl_2/MeOH) had potent antifungal activity against the mycelial growth of *Magnaporthe oryzae*. The active fraction (F2) was further fractionated over silica gel using CH_2Cl_2/MeOH (100:1) to give compounds **1** (356 mg) and **2** (5.0 mg).

Structural identifications of the bioactive metabolites were made on the basis of the spectroscopic analysis. The electrospray ionization mass spectrometry (ESI−MS) spectra were collected on a Mariner Mass 5304 instrument. The ^1H and ^{13}C nuclear magnetic resonance (NMR) data were acquired on a Bruker AVANCE-400 (Bruker,

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Switzerland) spectrometer at 400 MHz, and the chemical shifts were obtained in δ (ppm) by referring to the solvent signal and tetramethylsilane (TMS) as internal standards.

***In Vitro* Effect on Mycelial Growth of Phytopathogenic Fungi.** Antifungal bioactivities against mycelial growth of phytopathogenic fungi were performed by the agar medium assay as described previously.[11] Tested samples were dissolved in aqueous solution [composed of 1% dimethyl sulfoxide (DMSO) and 4% Tween-80] to the concentration of 100 μg/mL. Cycloheximide was used as the positive comparison. Solutions of serial concentrations of chemicals were mixed with liquid potato dextrose agar in a Petri dish (9 cm diameter). After inoculation of the fungal mycelia onto the center of solidified medium, the dishes were incubated in the dark at 28 °C. When the fungal mycelium reached the edges of the control dishes, the antifungal activities were calculated. The formula for counting the percentage of growth inhibition was shown as follows:

$$\text{inhibition}\ (\%) = (1 - D_a/D_b) \times 100$$

where D_a was the diameter of the growth zone in the experimental dish (mm) and D_b was the diameter of the growth zone in the control dish (mm). The IC_{50} values (the concentration inhibiting 50% of the mycelium growth) were calculated by probit analysis.

Spore Germination Assay. The inhibitory activity of the metabolite **1** to spore germination of *M. oryzae* was assessed as reported previously with slight changes.[13] *M. oryzae* spores were obtained from oatmeal−tomato agar plates of 7-day-old cultures. The metabolite **1** was made to obtain solutions with serial concentrations in aqueous solution (composed of 1% DMSO and 4% Tween-80). Test compound solution (50 μL) was added to spore suspension (50 μL, containing 2.5×10^5 spores). From this, aliquots of 40 μL of spore suspension from each were placed on separate glasses in triplicate. The control was treated with above aqueous solution alone. Slides containing spores were incubated in a moisture chamber at 28 °C for 12 h, after which approximately 100 spores from each of three replicates were examined under a light microscope to determine the percentage of germinated spores. The percentage of spore germination inhibition was calculated from mean values as

$$\text{inhibition}\ (\%) = 100 \times (A - B)/A$$

where A was the percentage of germinated spores in the control and B was the percentage of germinated spores in the sample. The IC_{50} value (the concentration inhibiting 50% of the spore germination) was calculated by probit analysis.

Evaluation of *in Vivo* Control Efficacy. Disease control efficacy of the metabolite **1** was evaluated against rice blast under growth chamber conditions. The *in vivo* bioassay was performed as described previously with a slight modification.[14] Briefly, 30-day-old rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) plants of all of the treatments and control were transferred to the inoculation chamber 1 day before the inoculation to acclimatize to the new environment at 28 ± 2 °C with 12 000 lx of illumination for 16 h per day and relative humidity above 95%. In this first preventive treatment, the plant seedling were sprayed to runoff with aqueous Tween 80 containing purified compound (100 μg/mL). Control plants were treated with Tween 80 solution. After 24 h, the treated rice seedlings of the fourth-leaf stage were inoculated with *M. oryzae* by spraying with a spore suspension (2.5×10^5 spores/mL) of the fungus. Carbendazim was applied as a positive control. Each experiment included three replicates per treatment. In a separate curative test, the purified compound was sprayed at 24 h post-inoculation and incubated under the same conditions as described above. At 11 days after fungal inoculation, the disease index was assessed following the study by Prabavathy et al.[15] Preventive efficacy and curative efficacy were calculated using the following equation:

$$\text{preventive or curative efficacy}\ (\%) = 100 \times (A - B)/A$$

where A was the disease index of the control and B was the disease index tested sample.

Statistical Analysis. All experiments were performed in triplicate, and data were shown as mean values ± standard deviation. A least significant difference (LSD) test with a confidence interval of 95% was used to compare the means.

RESULTS AND DISCUSSION

Identification of the Strain BYB02. The colonies of strain BYB02 were dry, circular, and produced brown pigment on the Gause's agar medium. The substrate and aerial mycelia were flourishing and branched, and the spore chains were white to gray. In comparison of cultural characteristics to those of actinomycetic species described in Bergey's Manual of Systematic Bacteriology, it suggested that the isolate belonged to the genus *Streptomyces*. A 1408 base pairs (bp) strain of the 16S rRNA gene (accession number JQ700298) was amplified from the strain BYB02. The BLAST matching analysis showed the 16S rRNA gene sequence of the strain had a high similarity (99.7%) to that of *Streptomyces canus* NRRL B-1989. Therefore, we treated this strain as *S. canus*, which was reported previously to produce several antibiotics, such as telomycin,[16] amphomycin,[17] and kanamycin.[18] The fungal strain BYB02 was deposited in the China Center for Type Culture Collection as CCTCCAA2012008.

Structure Elucidation of the Bioactive Metabolites. Bioassay-guided separation of the components in the ethyl acetate extract of *S. canus* BYB02 afforded two main bioactive metabolites (Figure 1) characterized by spectroscopic analyses,

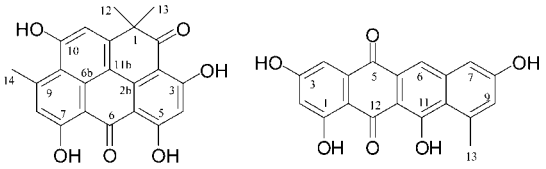

Figure 1. Chemical structures of compounds **1** and **2**.

including ESI−MS and NMR, and comparisons to the evidence described in the literature.[19,20] The constituents were identified as resistomycin (**1**) and tetracenomycin D (**2**) based on the following data.

Resistomycin (**1**): orange powder. ESI−MS: m/z $[M - H]^-$ 375 (calcd for $C_{22}H_{15}O_6$ 375.0869), $[2M - H]^-$ 751. ^{1}H NMR (DMSO-d_6) δ: 1.58 (6H, s, H-12 and H-13), 2.93 (3H, s, H-14), 6.38 (1H, s, H-4), 7.08 (1H, s, H-8), 7.27 (1H, s, H-11), 11.86 (1H, s, 10-OH), 14.05 (1H, s, 7-OH), 14.36 (1H, s, 5-OH), 14.57 (1H, s, 3-OH). ^{13}C NMR (THF-d_8) δ: 203.3(C-2), 184.2 (C-6), 169.6 (C-3), 169.0 (C-5), 167.3 (C-7), 160.3 (C-10), 150.9 (C-11a), 150.0 (C-9), 138.6 (C-2b), 127.8 (C-6b), 118.3 (C-8), 113.0 (C-9a), 107.6 (C-11), 105.6 (C-11b), 105.4 (C-6a), 104.8 (C-5a), 101.5 (C-2a), 99.1 (C-4), 44.5 (C-1), 27.8 (C-14), 26.5 (C-12 and C-13).

Tetracenomycin D (**2**): red powder. ESI−MS: m/z $[M - H]^-$ 335 (calcd for $C_{19}H_{11}O_6$ 335.0556), $[2M - H]^-$ 671. ^{1}H NMR (DMSO-d_6) δ: 2.83 (3H, s, H-13), 6.58 (1H, s, H-2), 7.00 (1H, s, H-7), 7.14 (1H, s, H-4), 7.19 (1H, s, H-9), 7.93 (1H, s, H-6), 10.62 (1H, s, 8-OH), 11.24 (1H, s, 3-OH), 12.24 (1H, s, 1-OH), 14.66 (1H, s, 11-OH).

***In Vitro* Effect on Mycelial Growth of Phytopathogenic Fungi.** The effects of metabolites **1** and **2** against mycelial growth of eight phytopathogenic fungi were evaluated *in vitro* (Table 1). The results showed that metabolite **1**

Table 1. IC_{50} Values of Compounds 1 and 2 against the Test Phytopathogens (in μg/mL)[a]

phytopathogen	1	2	cycloheximide[b]
M. grisea	3.8 ± 0.6	>25 ± 0	2.3 ± 0.1
V. mali	1.1 ± 0.3	>25 ± 0	0.3 ± 0
G. sanbinetti	>100 ± 0	N/T[c]	3.3 ± 0.2
R. solani	>100 ± 0	N/T	0.3 ± 0
D. gregaria	>100 ± 0	N/T	0.9 ± 0.1
F. oxysporum f. sp. *cucumerinum*	>100 ± 0	N/T	4.9 ± 0.2
F. oxysporum f. sp. *mornordicae*	>100 ± 0	N/T	10.6 ± 1.0

[a]Values represent the mean of three replications ± standard deviation. [b]Cycloheximide was co-assayed as a positive control. [c]N/T = not tested.

possessed significant antifungal activities against Ascomycota, such as *M. grisea* (IC_{50} = 3.8 μg/mL) and *Valsa mali* (IC_{50} = 1.1 μg/mL), which were comparable to those of referenced cycloheximide, with IC_{50} values of 2.3 and 0.3 μg/mL, respectively. However, it weakly inhibited the growth of *Rhizoctonia solani*, *Gibberella sanbinetti*, *Dothiorella gregaria*, *Fusarium oxysporum* f. sp. *cucumerinum*, and *Fusarium oxysporum* f. sp. *mornordicae* (IC_{50} > 100 μg/mL), which belonged to the microorganisms of Deuteromycotina. In general, compound **2** exhibited weak activities against *M. grisea* and *V. mali* (IC_{50} > 25 μg/mL).

The antimicrobial activities of compound **1** have been reported previously.[19,20] However, to the best of our knowledge, this was the first report about its antifungal activities against phytopathogens. Our test results suggested that the members of Ascomycota were sensitive and the species of Deuteromycotina were relatively resistant to the metabolite **1**; several similar selectivities were encountered previously for the compounds isolated from the insect-associated *Streptomyces* sp.[21−23] Therefore, insect-derived microbes would be a promising lead for the development of novel special fungicides.

Spore Germination Assay. The effect of the metabolite **1** on the percentage of spore germination for *M. grisea* was shown in Figure 2. As the metabolite **1** concentration increased, a reduction in the percentage of spore germination was observed. The 50% inhibitory concentration (IC_{50} value) of the metabolite **1** was 5.55 μg/mL. Thus, the metabolite **1** showed pronounced reduction in spore germination for *M. grisea*.

Evaluation of *in Vivo* Control Efficacy. Disease control efficacy of the metabolite **1** against rice blast was shown in Figure 3. The results showed that metabolite **1** possessed significant preventive efficacy, which was higher than that of referenced carbendazim, with control efficacies of 66.8 and 58.7%, respectively. However, it exhibited weak curative activity against the rice blast (control efficacy < 25%).

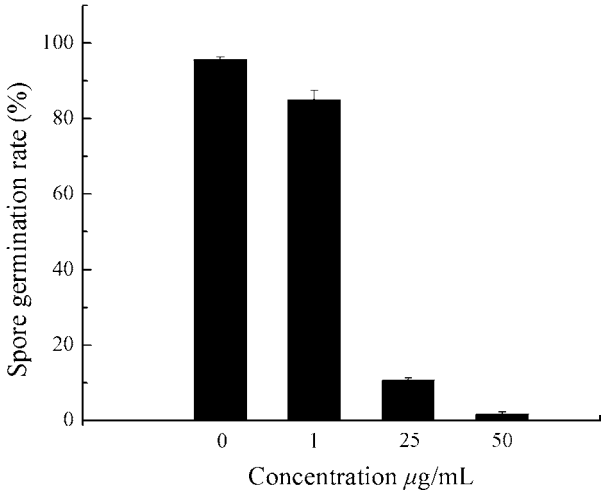

Figure 2. Effect of the metabolite **1** on the percentage of spore germination for *M. grisea*.

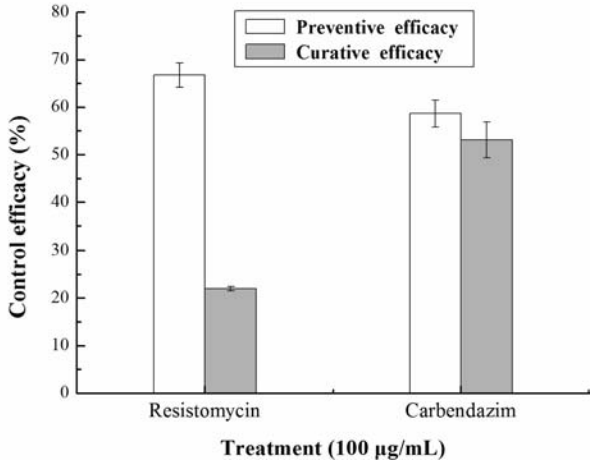

Figure 3. *In vivo* antifungal activities of the metabolite **1** and referenced carbendazim against the rice blast pathogen. Preventive/curative efficacy: the control efficacy of the compound that was sprayed on rice 1 day before/after inoculation.

Rice blast was the one of most important diseases affecting rice production in many rice-planting countries. To control this disease, the most usually used chemical agents were synthetic fungicides, which had caused several important problems, such as emergence of resistant pathogens, residual toxicity on food, and environmental pollution.[13] Microorganism-derived metabolites were generally recognized to be safer alternatives.[14] The present results showed that resistomycin from a termite-associated microbe had strong *in vitro* and *in vivo* antifungal activities against the rice blast pathogen. These findings strongly suggested that resistomycin can be used as a novel fungicide. However, further investigations are needed on resistomycin with regard to the disease control efficacy in various fields and side effects on nontarget organisms.

In conclusion, the natural products from the unexamined microorganisms were the important reservoirs of novel drugs. This study showed that the *S. canus* BYB02, a termite-associated actinomycete, could availably produce antifungal compounds, which indicated the feasibility that poorly or neglected investigated insect symbiontic microbes, such as the title strain, could be a good source for bioactive compounds. Grounded on the present results, resistomycin (**1**) could be an

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attractive lead compound for the development of a valuable antifungal agent.

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Notes

The authors declare no competing financial interest.

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