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Identification and Characterization of the Antifungal Substances of a Novel *Streptomyces cavourensis* NA4 S

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A new actinomycete strain NA4 was isolated from a deep-sea sediment collected from the South China Sea and showed promising antifungal activities against soilborne fungal pathogens. It was identified as *Streptomyces cavourensis* by morphological, physiological, and phylogenetic analyses based on its 16S rRNA gene sequence. The main antifungal components were isolated and identified from the fermentation culture as bafilomycins B1 and C1. These compounds exhibited significant antifungal activities and a broad antifungal spectrum. The results suggest that the *Streptomyces cavourensis* NA4 and bafilomycins B1 and C1 could be used as potential biocontrol agents for soilborne fungal diseases of plants.

Keywords: *Streptomyces cavourensis*, bafilomycins, antifungal activity, fungicide, marine actinomycete

Plant diseases have resulted in massive losses of agricultural produce [18]. In particular, soilborne plant diseases caused by *Fusarium oxyporum* and *Rhizoctonia solani etc.* have resulted in huge economic losses globally [1, 5]. Therefore, synthetic fungicides such as carbendazim and thiophanate-methyl have been extensively used. However, the long-term use of such chemical fungicides has not only resulted in unintended side effects on the environment and pesticide residues on agricultural products, but also increased pathogen resistance [18]. As a result, the demand for safe and efficient biological pesticides is growing continually [6]. In order to avoid generating cross-tolerance with original organic fungicides, the aim of the development of biocontrol agents is to target other mechanisms for plant protection [6, 14] .

Marine microorganisms are an important source for the development of new biocontrol agents owing to their very rich and varied biodiversity as well as rich and unique metabolites. A variety of marine microbial antagonists and their metabolites have been reported to control several different pathogens on various economic crops and vegetables [8, 10, 12, 16, 17]. Our laboratory is committed to developing marine microorganism-derived fungicides that can effectively prevent and control the wilt caused by *F. oxyporum* and *R. solani*-based pathogenic plant fungi [3].

As part of our ongoing research on biocontrol agents from marine actinomycetes, a new actinomycete strain (NA4) was isolated from a sediment collected at the depth

of 1,464 m from the South China Sea and was screened with a strong inhibitory effect on various fungal phytopathogens, including *Fusarium* spp., *R. solani, etc.* (Fig. S1). The strain NA4 was deposited at the China Center for Type Culture Collection with the number CCTCC M 2012530. It produced extensively branched substrate mycelia without diaphragms and fragments. Scanning electron microscopy revealed rectiflexibile chains borne on aerial hyphae, and the spore chains were relatively long, including about 50 spores with smooth surfaces and an elliptical shape (Fig. S2). These characteristics supported strain NA4 belonging to the genus *Streptomyces*.

An almost complete 16S rRNA gene sequence (1,344 bp) of strain NA4 was obtained and submitted to the GenBank Database with the accession number KF682222. Sequence analysis showed the 16S rRNA gene sequence of NA4 to be most similar to those of *Streptomyces cavourensis* NBRC13026^T, *Streptomyces albolongus* NBRC13465^T, and *Streptomyces celluloflavus* NBRC13780^T, with sequence identities of 100.0%, 99.93%, and 99.93%, respectively. Results of the phylogenetic analysis showed that NA4 was in the same branch on the cladogram with the three previously mentioned *Streptomyces*, suggesting a close genetic relationship (Fig. 1). A comparison of the phenotype characteristics indicated that the spores of *S. albolongus* were long columnar shaped, positive for tyrosinase, and generated black pigment, but negative using arabinose [11]. *S. celluloflavus* produced spirales-type spore chains, was negative for tyrosinase and had no pigment production [11]. Only *S. cavourensis* could use mannitol as the sole carbon source, and this species produced black pigment but was negative for tyrosinase (Tables S1 and S2). While the characteristics of strain NA4 was consistent with that of *S. cavourensis*, based on the comprehensive phenotype and phylogenetic analysis, strain NA4 was identified as *S. cavourensis*.

Moreover, two main antifungal metabolites **1** and **2** were isolated and purified from the fermentation culture of NA4 using HP20 macroporous adsorption resin, flash

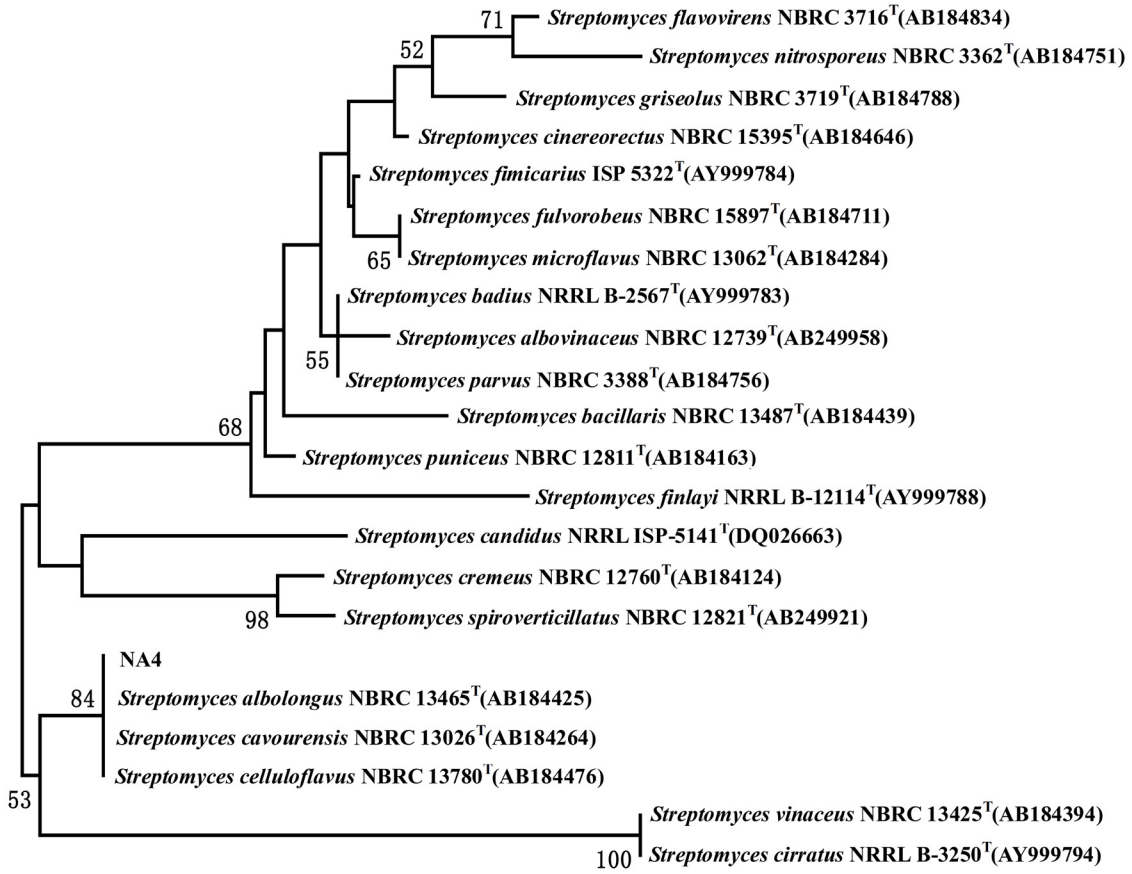

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain NA4 in the genus *Streptomyces*.

Bootstrap values (expressed as percentages of 1,000 replications) of above 50% are shown at the branch points. Bar, 0.001 sequence divergence.

chromatography over silica gel, and preparative thin layer chromatography. Compound **1** was obtained as a white amorphous powder. Its HRESIMS exhibited a $[M+Na]^+$ ion at *m/z* 743.4006 (Fig. S3), corresponding to a molecular formula of $C_{39}H_{60}O_{12}$, with 10 degrees of unsaturation. The ^1H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) spectra pointed to a bafilomycin derivative skeleton (Figs. S4 and S5). The structure of **1** (Fig. 2) was further elucidated to be bafilomycin C1 by 2D-NMR analyses including HSQC, ^1H–^1H COSY, and HMBC experiments (Fig. S6). Compound **2** was obtained as a yellow amorphous powder. The HRESIMS data (*m/z* 838.4345 $[M+Na]^+$) of **2** established the molecular formula to be $C_{44}H_{65}NO_{13}$ (Fig. S7). Moreover, compound **2** (Fig. 2) was identified as bafilomycin B1 by NMR spectrometry data (Figs. S8–S11). It should be noted that the signal of C8 (δ 39.4) was hidden in the solvent peak of DMSO, and the signals of the four carbon atoms in the α,β-unsaturated cyclopentanone ring had disappeared in the ^{13}C NMR spectra of compounds **1** and **2**, respectively. The structures of these compounds were hard to be certainly determined relying on 1D-NMR and HRESIMS data. Therefore, all these signals of C and H could be correctly and completely attributed only in combination with 2D-NMR. All the proton and carbon signals of these compounds are assigned in Table S3.

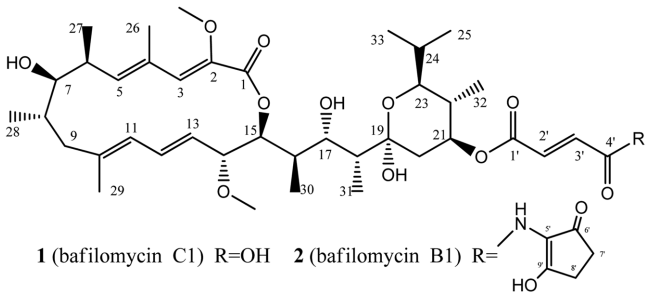

Fig. 2. Chemical structures of compounds 1 and 2.

The antifungal activities of these compounds were evaluated using the paper-agar disk diffusion assay. The result indicated that the two compounds showed significant inhibitory activities against a variety of *Fusarium* spp. and *R. solani*, while being inactive against *Setosphaeria turcica*. Their antifungal activities were concentration-dependent. The highest inhibitory activity was observed for *Fusarium oxysporum* f. sp. *corylus chinensis* and each piece of paper held a 10 μg sample with its inhibition zone diameters being 15.16 mm and 19.93 mm, respectively (Table 1). It was notable that these bafilomycins showed better antifungal activities against *Fusarium* spp. than amphotericin B as the control drug. Specifically, the *F. oxysporum* species complex comprises different formae speciales (f. sp.) that exhibit high host specificity [9]. It is a devastating pathogen that affects many important food and vegetable crops and is a major source of loss to farmers worldwide. Unfortunately, no effective strategy to control the pathogen is currently available. To the best of our knowledge, this is the first report on the significant inhibitory activity of bafilomycins B1 and C1 against *Fusarium* spp. [4, 15]. Based on a comparison of the antifungal activities and antifungal

Table 1. *In vitro* antifungal activity of compounds 1 and 2 against a panel of plant pathogens.

Pathogenic fungi	Compound 1			Compound 2			Amphotericin B	Control
	10 μg	5 μg	2.5 μg	10 μg	5 μg	2.5 μg	10 μg	
Fusarium oxysporum f. sp. *cucumerinum*	10.45 ± 0.13	10.05 ± 0.29	11.07 ± 0.60	11.36 ± 0.43	10.66 ± 0.55	10.58 ± 0.54	11.86 ± 0.29	8
Fusarium oxysporum f. sp. *corylus chinensis*	15.16 ± 1.00	12.53 ± 0.77	11.80 ± 0.40	19.93 ± 0.59	16.51 ± 0.99	14.33 ± 1.15	15.09 ± 0.93	8
Fusarium oxysporum f. sp. *cucumis melo* L.	10.88 ± 0.83	8.47 ± 0.42	8	14.79 ± 1.00	12.48 ± 1.46	10.32 ± 0.29	8	8
Fusarium oxysporum f. sp. *vasinfectum*	13.64 ± 0.92	10.19 ± 0.63	9.08 ± 0.18	14.40 ± 1.82	10.65 ± 0.58	9.93 ± 0.77	13.17 ± 0.49	8
Fusarium graminearum	8	8	8	15.89 ± 0.40	12.00 ± 0.53	12.52 ± 0.63	8	8
Fusarium solani	9.70 ± 0.40	8.61 ± 0.53	8	10.12 ± 0.46	8	8	9.47 ± 0.23	8
Fusarium moniliforme	10.42 ± 0.38	8.35 ± 0.30	8	11.63 ± 1.10	9.07 ± 0.10	9.69 ± 0.57	8	8
Rhizoctonia solani	10.81 ± 0.36	10.35 ± 0.28	9.51 ± 0.45	12.52 ± 0.81	8.72 ± 0.25	8	13.19 ± 0.25	8
Botrytis cinerea	11.45 ± 1.23	9.20 ± 1.06	8	10.84 ± 0.92	9.72 ± 0.66	9.23 ± 0.38	10.72 ± 0.62	8
Setosphaeria turcica		8	8	8	8	8	16.45 ± 0.66	8

Diameter of inhibition zone represents the mean ± SD of three independent readings; SD: standard deviation.
Diameter of paper disks: 8 mm.

spectrum of the two compounds, compound **2** showed a stronger activity and a wider antifungal spectrum. It suggested that the α,β unsaturated cyclopentanone ring helped to improve the antimicrobial activity of bafilomycin B1. In other words, these bafilomycins may have a role in the biocontrol of different fungal phytopathogens.

HPLC analysis was applied for the standard curves based on the standard bafilomycin B1 and bafilomycin C1 at different concentrations, and we determined the contents of bafilomycins B1 and C1 from fermentation supernatants (7.88 mg/l and 2.78 mg/l, respectively). The contents of bafilomycin B1 and bafilomycin C1 were 15.58 mg/l and 3.73 mg/l from mycelia, respectively. Each liter of *Streptomyces cavourensis* NA4 fermentation broth yielded a total of 23.45 mg of bafilomycin B1 and 6.60 mg of bafilomycin C1. To the best of our knowledge, this is the highest reported content of bafilomycins produced from a wild-type strain [2, 4, 7, 13, 15, 19]. The yield can be further improved through induced breeding, optimized culture conditions, and composition of the culture medium. In addition, only two bafilomycins, bafilomycin D and hygrobafilomycin, have been reported from *S. cavourensis* [17]. This is the first report that *S. cavourensis* produced bafilomycins B1 and C1. Therefore, *S. cavourensis* NA4 could be used as a bafilomycin producer for the control of fungal diseases.

In this study, we described the marine-derived *S. cavourensis* strain NA4, which exhibits potent inhibitory activities against *Fusarium* spp., *R. solani*, and *Botrytis cinerea*, and its antagonistic effects are mainly due to the production of antifungal antibiotics, bafilomycins B1 and C1. The present results suggest that *S. cavourensis* NA4 could be used as a potential biocontrol agent for soilborne fungal diseases of plants and a novel producer for bafilomycins; and at the same time, bafilomycins B1 and C1 could be two promising lead compounds for the development of valuable fungicides.

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References

1. Anees M, Edel-Hermann V, Steinberg C. 2010. Build up of patches caused by *Rhizoctonia solani*. *Soil Biol. Biochem.* **42:** 1661-1672.
2. Carr G, Williams DE, Diìaz-Marrero AR, Patrick BO, Bottriell H, Balgi AD, *et al.* 2009. Bafilomycins produced in culture by *Streptomyces* spp. isolated from marine habitats are potent inhibitors of autophagy. *J. Nat. Prod.* **73:** 422-427.
3. Chen L, Wang N, Wang X, Hu J, Wang S. 2010. Characterization of two anti-fungal lipopeptides produced by *Bacillus amyloliquefaciens* SH-B10. *Bioresour. Technol.* **101:** 8822-8827.
4. Frändberg E, Petersson C, Lundgren LN, Schnürer J. 2000. *Streptomyces halstedii* K122 produces the antifungal compounds bafilomycin B1 and C1. *Can. J. Microbiol.* **46:** 753-758.
5. Fravel D, Olivain C, Alabouvette C. 2003. *Fusarium oxysporum* and its biocontrol. *New Phytol.* **157:** 493-502.
6. Kim B, Hwang B. 2007. Microbial fungicides in the control of plant diseases. *J. Phytopathol.* **155:** 641-653.
7. Li J, Lu C, Shen Y. 2010. Macrolides of the bafilomycin family produced by *Streptomyces* sp. CS. *J. Antibiot.* **63:** 595-599.
8. Peng JN, Shen XY, El Sayed KA, Dunbar DC, Perry TL, Wilkins SP, Hamann MT. 2003. Marine natural products as prototype agrochemical agents. *J. Agric. Food Chem.* **51:** 2246-2252.
9. Poli A, Gilardi G, Spadaro D, Gullino M, Garibaldi A. 2012. Molecular characterization of *Fusarium oxysporum* f.sp. *cichorii* pathogenic on chicory (*Cichorium intybus*). *Phytoparasitica* **40:** 383-391.
10. Shanzhao J, Ling L, Zhaopu L, Xiaohua L, Hongbo S, Jiayao C. 2013. Characterization of marine *Pseudomonas* spp. antagonist towards three tuber-rotting fungi from Jerusalem artichoke, a new industrial crop. *Ind. Crops Prod.* **43:** 556-561.
11. Silva L, Taketani R, Melo I, Goodfellow M, Zucchi T. 2013. *Streptomyces araujoniae* sp. nov.: an actinomycete isolated from a potato tubercle. *Antonie Van Leeuwenhoek* **103:** 1235-1244.
12. Tareq FS, Kim JH, Lee MA, Lee HS, Lee JS, Lee YJ, Shin HJ. 2013. Antimicrobial gageomacrolactins characterized from the fermentation of the marine-derived bacterium *Bacillus subtilis* under optimum growth conditions. *J. Agric. Food Chem.* **61:** 3428-3434.
13. Uyeda M, Kondo K, Ito A, Yokomizo K, Kido Y. 1995. A new antiherpetic agent produced by *Streptomyces* sp. strain no. 758. *J. Antibiot.* **48:** 1234.
14. Wang X, Radwan MM, Taraìwneh AH, Gao J, Wedge DE, Rosa LH, *et al.* 2013. Antifungal activity against plant pathogens of metabolites from the endophytic fungus *Cladosporium cladosporioides*. *J. Agric. Food Chem.* **61:** 4551-4555.
15. Werner G, Hagenmaier H, Drautz H, Baumgartner A, Zahner H. 1984. Metabolic products of microorganisms. 224. Bafilomycins, a new group of macrolide antibiotics.

Production, isolation, chemical structure and biological activity. *J. Antibiot.* **37:** 110-117.
16. Xie L, Jiang S, Zhu H, Sun W, Ouyang Y, Dai S, Li X. 2008. Potential inhibitors against *Sclerotinia sclerotiorum*, produced by the fungus *Myrothecium* sp. associated with the marine sponge *Axinella* sp. *Eur. J. Plant Pathol.* **122:** 571-578.
17. Xu WP, Zhang DJ, Si CC, Tao LM. 2013. Antifungal macrolides from *Streptomyces cavourensis* YY01-17. *Chem. Nat. Compounds* **49:** 988-989.
18. Yoon MY, Cha B, Kim JC. 2013. Recent trends in studies on botanical fungicides in agriculture. *Plant Pathol. J.* **29:** 1-9.
19. Zhang DJ, Wei G, Wang Y, Si CC, Tian L, Tao LM, Li YG. 2011. Bafilomycin K, a new antifungal macrolide from *Streptomyces flavotricini* Y12-26. *J. Antibiot.* **64:** 391-393.