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Communication

Isolation and Structure Elucidation of Peronosporomycetes Hyphal Branching-Inducing Factors Produced by *Pseudomonas jessenii* EC-S101

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***Pseudomonas jessenii* EC-S101 produced hyphal branching-inducing and mitosis-accelerating factors active towards Peronosporomycetes, *Aphanomyces cochlioides* hyphae. In searching for the active substances, EtOAc-solubles extracted from EC-S101-cultured solid medium were fractionated under the guidance of a paper disc assay using an *A. cochlioides* mycelium. Two active substances were subsequently isolated and the structure was elucidated by spectroscopic analysis to be (+)-4,5-didehydroacaterin (1) and 3-[(1*R*)-hydroxyhexyl]-5-methylene-2(5*H*)-furanone (2), both of which accelerated the mitotic process of *A. cochlioides* hyphae along with excessive branching at 1.0 μg per disc. These compounds are likely to affect the morphophysiological development of certain eukaryotic organisms in the terrestrial ecosystem.**

Key words: *Aphanomyces cochlioides*; excessive hyphal branching-inducing factor; γ-lactone derivatives; *Pseudomonas jessenii*

In our screening of functional rhizospherous microorganisms, interference among microfloral components was occasionally observed. Among a total of 157 bacteria isolated from the rhizoplanes of plants, eight strains inhibited the hyphal growth of a phytopathogenic Peronosporomycetes, *Aphanomyces cochlioides* AC-5, in a dual culture assay.[1] *Pseudomonas jessenii* strain EC-S101, one of the most active isolates, induced unique excessive branching in the hyphae by continuous apical bifurcation toward the bacterial colonies. Using Hoechst dye 33258 for fluorescent staining of nuclei, it was found that acceleration of mitosis also occurred in the branching hyphae.[2] Chemical principles were expected to be produced and released from strain EC-S101.

EtOAc-soluble extracts from culture fluids of *P. jessenii* strain EC-S101 induced similar excessive branching in *A. cochlioides* hyphae. Using paper disc assay for excessive branching induction in *A. cochlioides* AC-5, chemical spots detected at R_f 0.50 in $CHCl_3$–MeOH 24:1 were found to be the active principle. By silica gel column chromatography and subsequent HPLC, two active substances were consequently purified, each as a colorless syrup (see the materials and methods section below). Physicochemical data for major compound **1** showed good accordance with those of (+)-4,5-didehydroacaterin,[3] which has been isolated as a precursor compound of acaterin (**3**), an antibiotic of *Pseudomonas* sp. A92.[4] Since the optical rotations of **1** and the authentic compound agreed approximately,[3] active principle **1** was identified as (+)-4,5-didehydroacaterin.

On the other hand, minor compound **2** also possessed UV λ_{max} at 262 nm in MeOH, same as **1** did, suggesting the presence of α,β,γ,δ-unsaturated carbonyl moiety in the molecule. Compound **2** showed a molecular ion at m/z 196 in FD-MS, and its molecular formula ($C_{11}H_{16}O_3$) was determined by EI-HR-MS, with an unsaturation index of 4. Because compound **2** exhibited the same mass fragmentation patterns as those of **1** (*e.g.*, $M^+ - H_2O$ and $M^+ - 29$) and base peaks (m/z 125, 100%) in EI-MS, it is probable that **2** is a homologous compound of **1**, different in the length of the alkyl side chain.

In ^{1}H- and ^{13}C-NMR analyses of compound **2**, including ROESY, HMQC and HMBC, the assignment of all proton signals was completed. ^{1}H–^{1}H-COSY revealed the proton coupling sequences of **2**. An isolated pair of methylene signals (δ_H 1.8 and 1.7, both *m*) were coupled to a hydroxylated methine proton (δ_H 4.60, *br. t*-like, $J = 6$ Hz) at C-2′, which also showed an allyl coupling with the olefinic proton (δ_H 7.20, *d*, $J = 0.5$ Hz) at C-3 on the α,β,γ,δ-conjugated exomethylenelactone moiety. One exomethylene proton signal (δ_H 5.21, C-5-H_b) showed a clear ROESY cross peak to the olefinic proton at C-3. Consequently, major compound **2** was determined to be 3-(1-hydroxyhexyl)-5-methylene-2(5*H*)-furanone (Fig. 1), a novel homolog of **1**. Since

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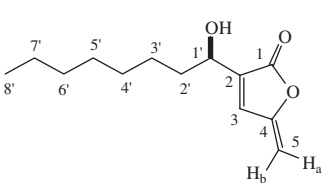
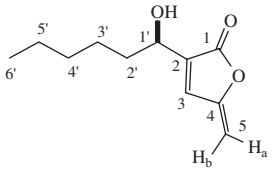
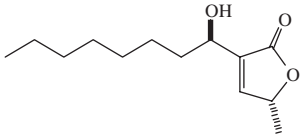


(+)-4,5-Didehydroacaterin (**1**)



3-[(1*R*)-Hydroxyhexyl]-5-methylene-2(5*H*)-furanone (**2**)



(+)-Acaterin (**3**)

Fig. 1. Chemical Structures of Excessive Hyphal Branching-Inducing Factors **1** and **2** and a Related Natural Product **3**.

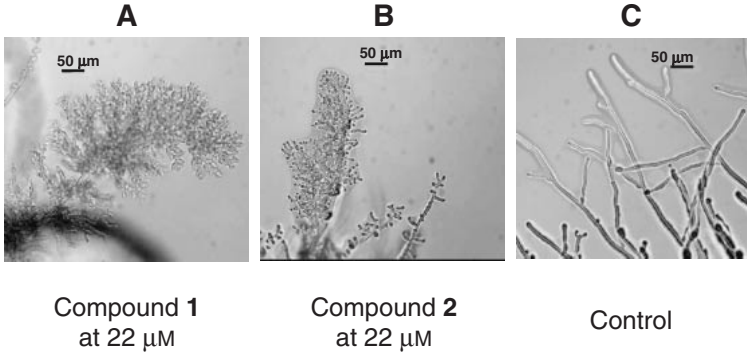


Fig. 2. Excessive Branching Induced in *Aphanomyces cochlioides* Hyphae Grown on an Agar Medium That Contained a Purified Active Principle. Compounds **1** and **2** at a concentration of 22 μM in potato-dextrose agar medium induced fractal hypal branching (A and B, *cf.* control in C). After inoculation with an *A. cochlioides* mycelial disc, incubation was allowed at 25 °C for 3 d in the dark. The bars in the photographs are 50 μm.

compound **2** from *P. jessenii* showed dextrorotatory (+22.4°), absolute configuration of **2** at the C-1′ chiral carbons was probably *R*.[3,5]

Acaterin (**3**) is an acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, a downstream product derived from **1** in the acetogenin-pathway of *Pseudomonas* sp. A92.[4] We have also determined by GC that compound **3** was produced by *P. jessenii* EC-S101. Both chemical substances **1** and **2** induced excessive branching of *A. cochlioides* hyphae along with acceleration of mitosis at a minimal concentration of 4–22 μM in the medium (Fig. 2), or at 1 μg per disc (Fig. 3), but compound **3**, which accumulated in the culture fluids of *P. jessenii* EC-S101 at a concentration similar to **1** showed neither hyphal branching-induction nor mycelial growth suppression.

As an effect similar to induction of excessive branching, this has also been reported in *Botrytis cinerea* exposed to cytochalasins A and D (inhibitors for glucose transport and actin polymerization),[6] and indeed, *A. cochlioides* AC-5 showed morphologically similar patterns when it was treated with cytochalasin A at 25 μg/disc. Recently, it was found that Ca^{2+} regulators such as calcineurin induced excessive apical branching in the growing hyphae of *Neurospora crassa* and *Aspergillus fumigatus*, as those antifungal compounds do.[7–9] Association of cytoplasmic Ca^{2+} concentration with apical branching induction was also demonstrated by means of calcimycin (calcium ionophore A23187) treatment of *A. cochlioides* hyphae at 2.5 μg/disc.[1]

Observation under confocal laser scanning microscopy (CLSM) of nuclei in excessively branched hyphae (Fig. 3B, C) showed that hyphal cell mitosis was accelerated but compounds **1** and **2** did not show any acute toxicity. The older hyphae, however, showed nuclei degradation spreading blue fluorescence in the cystoplasma. On the other hand, F-actin observation indicated that apical tips of the hyphae affected by compound **1** were rich in the plaque-form of F-actin, which generally emerges at the stationary stage of hyphae rather than the tips of normally growing hyphae

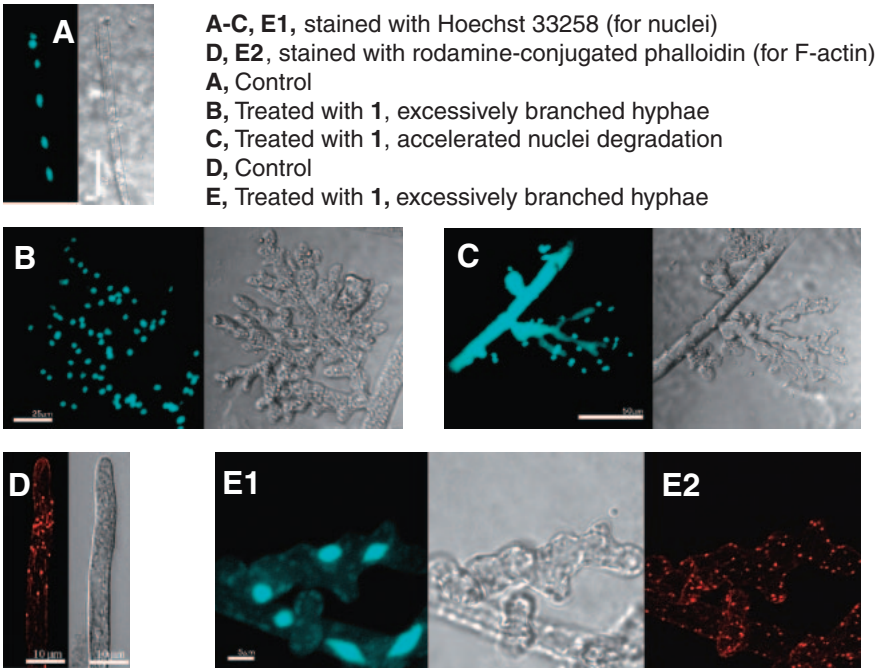


Fig. 3. Induction of Mitosis and Acceleration of Senescence in Excessively Branched *Aphanomyces cochlioides* Hyphae.

At a concentration of 1 µg per disc, compound **1** induced excessive hyphal branching along with mitotic acceleration. A, control; B and C, affected hyphae after 2 d of incubation. (B) are at relatively early stage of hyphal branching. Compared with control (A), showing oval shaped nuclei, the nuclei in branched hyphae in (B) are rather spherical in shape. Stimulation of hyphal cellular mitosis is also apparent in (B), whereas (C) is at a relatively late stage of the hyphal branching induction. At this stage, degradation of nuclei is apparent, showing acceleration of hyphal senescence. D, control for F-actin staining; E, status of nuclei (E1) and F-actin (E2) in excessively branched hyphae. The CLSM photographs in dark view (left) were for detecting fluorescence, while those in daylight (right) were for reference. Bars in (A) and (B) are 25 µm, whilst those in (C), (D), and (E) are 50, 10, and 5 µm respectively.

(Fig. 3D, E). Altogether, these compounds are most likely to be signaling mimics rather than fatal toxic compounds. In fact, most of the other anti-Peronosporomycetes chemicals, such as hymexazol, simply killed the approaching hyphae,[1] whilst cytochalasins that inhibit F-actin polymerization may affect F-actin assembly in developing hyphal cells, leading to excessive branching induction similar to that by **1** and **2**.

The fact of suppression of infection with *A. cochlioides* during contact with *P. jessenii* EC-S101 in a pot experiment[1] suggests that the EC-S101-colonized rhizoplane of spinach seedlings[2] produces compounds **1** and **2** as effective chemical agents to defend host plants from phytopathogenic Peronosporomycetes. Thus *P. jessenii* has a relationship with host plant roots *via* compounds **1** and **2**, although the ecological significance of compounds **1** and **2** is not yet clear.

In some cases, proteobacteria induce cell differentiation in eukaryotic organisms. One example is the case of *Xanthomonas campestris* and *Candida albicans*, a yeast showing dimorphic transition. When they are dual-cultured, a quormone-mimic, *cis*-11-methyl-2-dodecenoic acid, produced by *X. campestris* inhibits the hyphal development of *C. albicans*.[10] Also, in the relationship between an alga-epiphytic bacterium and sea lettuce (*Monostroma oxyspermum*), an active principle, thallusin, produced by a marine bacterium from the Cytophaga-Flavobacterium-Bacteroides group, plays a role in inducing a foliaceous shape in the alga.[11] Given observations described above and the *P. jessenii-A. cochlioides* relationship, there is likely to be similar phenomena in which certain prokaryotes affect the morphophysiological development of eukaryotic organisms.

Compounds **1** and **2** possessed the same carbon skeleton as that of a bromo-furanone isolated from a red marine alga.[12] Because such bromination is known to be a unique character of marine natural products,[13] it is tempting to speculate that bromine-free furanone derivatives **1** and **2** are terrestrial compounds. Importantly, this brominated natural product is now widely accepted as a quorum-sensing signal mimic and an inter-kingdom cross-talk signal compound,[14] and indeed, it suppresses or promotes several common proteobacteria along with activation of quormone-related genes.[15] Therefore, compounds **1** and **2**, which are positioned in the bromo-furanone in the terrestrial ecosystem, may act as quorum-sensing signal mimics. The cross-talk signal-like roles of **1** and **2** in the terrestrial ecosystem, together with the behaviors of *P. jessenii* EC-S101, will be reported elsewhere. The materials and methods section follows:

Original source of the antagonistic bacterium. P. jessenii strain EC-S101, which induced excessive branching in *A. cochlioides* hyphae, was originally isolated from the rhizosphere of spinach grown at the exper-

imental farm of the Graduate School of Agriculture of Hokkaido University in Sapporo, Japan. The isolate was identified by means of sequence determination in the region of the 16S rRNA gene, and sequences were amplified using 27F and 1525R universal primers for PCR. The sequence data (1,440 bp) were deposited at the DNA Data Bank of Japan (DDBJ) at the National Institute of Genetics, Mishima, Japan, and assigned accession no. AB190286.

Extraction of secondary metabolites. EC-S101 was pre-cultured at 25 °C in 100 ml of nutrient broth in a 300 ml-Erlenmeyer flask in a rotary shaker at 100 rpm for 24 h. The cultivated medium (total, 75 ml) was spread on the surface of 7.5-liter of potato dextrose agar medium in 250 of square dishes ($130 \times 95\,mm^2$, 30 ml each) at a thickness of 2.4 mm, and incubated at 28 °C in the dark for 24 h. The resulting plates were frozen at −20 °C, and subsequently defrosted agar medium was squeezed using a double-layered cheese cloth to collect culture fluid of 4.5-liter in volume. The culture fluid thus obtained was filtered with filter paper. The residual agar was again washed with 1.5-liter of deionized water and repeatedly filtered as described above. The filtrates and washings were combined and centrifuged (8,500 rpm at 4 °C for 18 min) and re-filtered using a Millipore filter (PTFE membrane, 0.4 mm, Millipore, Billerico, MA). The resulting supernatant (5.5-liter) was extracted with EtOAc (5-liter × 3), and the EtOAc layer thus obtained was dried over $MgSO_4$ and then concentrated in a vacuum to obtain 250 mg of syrup.

Fractionation and isolation of mycelial growth-inhibiting principles. The EtOAc solubles were applied to 1.9 cm i.d. of silica gel column chromatography (25 g of Wako-gel C60, Wako Pure Chemical Industries Co. Ltd., Osaka, Japan), and eluted with hexane–EtOAc 4:1 (90 ml, Fr-1–5), hexane–EtOAc 2:1 (90 ml, Fr-6–10) and 100% EtOAc (90 ml, Fr-11–15). Fractions Fr-5–12 (84.3 mg) showed inhibitory activity against mycelial growth of *A. cochlioides* AC-5, and all the active fractions were subjected to silica gel prep. TLC (20 cm × 20 cm plate) and developed in hexane–EtOAc 3:1. The active principles obtained were then subjected to HPLC (Inertsil C18-Prep-Sil, 6 mm i.d. × 250 mm, GL Sciences Inc., Tokyo) in CH_3CN–water 6:4 and detected at UV 254 nm, and two active compounds (**1** and **2**) were eventually obtained, at 30.0 mg and 8.0 mg respectively.

*Physicochemical properties of didehydroacaterin (**1**).* GC–MS *m/z* (%): 224 (M^+, 1.3), 206 ($M^+ - H_2O$, 12), 195 (16), and 125 ($M^+ - C_7H_{15}$, 100). EI-HR-MS: $C_{13}H_{20}O_3$ (found 224.1438, calcd. 224.1413). UV λ_{max} (MeOH): 262 nm. $[\alpha]_D$: +23.2° at 28 °C ($c = 7.8$, 10 mm cell length, in $CHCl_3$). ^{1}H- and ^{13}C-NMR signals of **1** showed accordance with those of didehydroacaterin.[3] *Physicochemical properties of 3-(1-hydroxyhexyl)-5-methylene-2(5H)-furanone (**2**).* GC–MS *m/z* (%): 196 (M^+, 4.8), 178 ($M^+ - H_2O$, 12), 167 (28), 126 (35), 125 ($M^+ - C_5H_{11}$, 100), 107 (11), 97 (33), 43 (19), 41 (11). EI-HR-MS: $C_{11}H_{16}O_3$ (found 196.1127, calcd. 196.1100). UV λ_{max} (MeOH): 262 nm. $[\alpha]_D$: +22.4° at 28 °C ($c = 7.8$, 10 mm cell length, in $CHCl_3$). The ^{1}H-NMR (270 MHz, in $CDCl_3$): 7.20 (*d*, $J = 0.5$ Hz, 3-H), 5.21 (*d*, $J = 2.6$ Hz, 5-Ha), 4.89 (*d*, $J = 2.6$ Hz, 5-Hb), 4.60 (*br. t*, $J = 6$ Hz, 1′-H), 1.8 (*m*, 2′-Ha), 1.7 (*m*, 2′-Hb), 1.5–1.2 (6H, *m*, 3′, 4′ and 5′-H_2), and 0.91 (3H, *br. t*, $J = 6$ Hz, 6′-H_3). ^{13}C-NMR (67 MHz, in $CDCl_3$): 169.0 (1-C), 153.5 (4-C), 138.1 (2-C), 136.1 (3-CH), 97.6 (5-CH_2), 67.1 (1′-CH), 35.7 (2′-CH_2), 31.6, 25.0 and 22.6 (3′, 4′ and 5′-CH_2), and 14.1 (6′-CH_3).

CLSM for nuclei observation. To detect whether mitosis was accelerated in the excessively branched hyphae of AC-5 upon exposure to compounds **1** and **2**, we stained with Hoechst 33258 for nuclei[1] (Sigma-Aldrich, St. Louis, MO) or rhodamine-conjugated phalloidin for F-actin (Invitrogen, Carlsbad, NM) the AC-5 hyphae that were grown on corn meal agar medium (3-d-old) and allowed them to interact with compound **1** or **2** charged on a paper disc at 1.0–2.5 μg per disc. The CLSM used in this experiment was a Zeiss CLSM LSM410 (Carl Zeiss, Oberkochen, Germany) equipped with an Axiovert 135M microscope with objective lenses containing magnifications/numerical apertures of 20/0.5 and 40/0.75. The sample preparation and observations were conducted as described in our previous paper.[2]

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References

1) Deora, A., Hashidoko, Y., Islam, M. T., and Tahara, S., Antagonistic rhizoplane bacteria induce diverse morphological alterations in Peronosporomycete hyphae during *in vitro* interaction. *Eur. J. Plant Pathol.*, **112**, 311–322 (2005).
2) Deora, A., Hashidoko, Y., Islam, M. T., Aoyama, Y., Ito, T., and Tahara, S., An antagonistic rhizoplane bacterium *Pseudomonas* sp. strain EC-S101 physiologically stresses a spinach root rot pathogen *Aphanomyces cochlioides*. *J. Gen. Plant Pathol.*, **72**, 57–64 (2006).
3) Sekiyama, Y., Araya, H., Hasumi, K., Endo, A., and Fujimoto, Y., Biosynthesis of acaterin: isolation of 4,5-didehydroacaterin and its conversion into acaterin. *Nat. Prod. Lett.*, **11**, 61–66 (1997).
4) Naganuma, S., Sakai, K., Hasumi, K., and Endo, A., Acaterin, a novel inhibitor of acyl-CoA: cholesterol acyltransferase produced by *Pseudomonas* sp. A92. *J. Antibiotics*, **45**, 1216–1221 (1992).

5) Ishigami, K., and Kitahara, T., Synthesis of all the four possible stereoisomers of acaterin, naturally occurring ACAT inhibitor, and the determination of its absolute configuration. *Tetrahedron*, **51**, 6431–6442 (1995).
6) Betina, V., Micekova, D., and Nemec, P., Antimicrobial properties of cytochalasins and their alteration of fungal morphology. *J. Gen. Microbiol.*, **71**, 343–349 (1972).
7) Grinberg, A., and Heath, B., Direct evidence for Ca^{2+} regulation of hyphal branch induction. *Fungal Genet. Biol.*, **22**, 127–139 (1997).
8) Prokisch, H., Yarden, O., Dieminger, M., Tropschug, M., and Barthelmess, I. B., Impairment of calcineurin function in *Neurospora crassa* reveals its essential role in hyphal growth, morphology and maintenance of the apical Ca^{2+} gradient. *Mol. Gen. Genet.*, **256**, 104–114 (1997).
9) Steinbach, W. J., Cramer, R. A., Jr., Perfect, B. Z., Asfaw, Y. G., Sauer, T. C., Najvar, L. K., Kirkpatrick, W. R., Patterson, T. F., Benjamin, D. K. Jr., Heitman, J., and Perfect, J. R., Calcineurin controls growth, morphology, and pathogenicity in *Aspergillus fumigatus*. *Eukaryot. Cell*, **5**, 1091–1103 (2006).
10) Wang, L.- H., He, Y., Gao, Y., Wu, J. E., Dong, Y.-H., He, C., Wang, S. X., Weng, L.-X., Xu, J.-L., Lay, L., Fang, R. X., and Zhang, L.-H., A bacterial cell-cell communication signal with cross-kingdom structural analogues. *Mol. Microbiol.*, **51**, 903–912 (2004).
11) Matsuo, Y., Imagawa, H., Nishizawa, M., and Shizuri, Y., Isolation of an algal morphogenesis inducer from a marine bacterium. *Science*, **307**, 1598 (2005).
12) De Nys, R., Wright, A., Koning, M., and Sticher, O., New halogenated furanones from the marine alga *Delisea pulchra* (cf. *fimbriata*). *Tetrahedron,* **49**, 11213–11220 (1993).
13) Bauer, W. D., and Teplitski, M., Can plants manipulate bacterial quorum sensing? *Aust. J. Plant Physiol.*, **28**, 913–921 (2001).
14) Ren, D., Sims, J. J., and Wood, T. K., Inhibition of biofilm formation and swarming of *Bacillus subtilis* by (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone. *Lett. Appl. Microbiol.*, **34**, 293–299 (2002).
15) Garson, M. J., Biosynthetic studies on marine natural products. *Nat. Prod. Rep.*, **6**, 143–170 (1988).