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Identification of Orfamide A as an Insecticidal Metabolite Produced by *Pseudomonas protegens* F6

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Supporting Information

ABSTRACT: The use of biosurfactants for agricultural crop protection has been gaining interest because they are generally biodegradable and environmentally friendly. In the present study, we identified an insecticidal biosurfactant produced by *Pseudomonas protegens* F6 (F6) and examined its use for aphid control. The growth of F6 was accompanied by increased aphid mortality and decreased water surface tension. Bioassay-guided chromatography coupled with instrumental analyses, nuclear magnetic resonance (NMR), and time-of-flight mass spectrometer (TOF MS) identified orfamide A as a major metabolite that showed insecticidal activity against green peach aphid (*Myzus persicae*). Orfamide A revealed a dose-dependent mortality against aphids, producing a LC_{50} value at 34.5 μg/mL, and caused a considerable decrease in the surface tension value of water, giving about 35.7 mN/m at 10 μg/mL. Laboratory and greenhouse mortality bioassays suggested that orfamide A may be applicable to control aphids in organic agriculture. This is the first report of orfamide A as an insecticidal metabolite against *Myzus persicae*.

KEYWORDS: *orfamide A, aphid, biosurfactant, biopesticide, Pseudomonas protegens F6*

INTRODUCTION

The green peach aphid *Myzus persicae* (Sulzer) is one of the most common aphids worldwide, and causes a significant reduction in crop production and quality. The leaf deformation and the spread of plant pathogens or viral diseases are typical effects of aphids on crops.[1,2] A number of conventional synthetic pesticides have been extensively used for aphid control, but their potential impacts on the environment and undesirable effects on nontarget organisms suggest the use of more environmentally friendly means. Thus, finding new alternative means is of interest to agricultural researchers.

The use of microbial metabolites in pest management has been growing in agriculture because they are generally more biodegradable and environmentally friendly than conventional pesticides. Biosurfactants are typical examples of microbial metabolites that show biological activity against pest insects and pathogens. Sophorolipids, produced by *Candida bombicola* and *Pseudomonas aeruginosa*, have been shown to have antimicrobial activity against pathogens that cause gray and softened spots on apple and tomato.[3,4] Sophorolipids appeared to significantly inhibit the growth of algal cells by perturbing their membranes.[5] Rhamnolipids produced by *Pseudomonas* strains were found to significantly inhibit the growth of zoosporic plant pathogens and harmful algal blooms.[6−8] Similarly, surfactin produced by *Bacillus subtilis* C1 appears to inhibit the growth of the blooming cyanobacterium *Microcystis aeruginosa*.[9] Much effort on biosurfactants as biocontrol agents has focused on the area of biological control of plant pathogens, but little effort has been made to determine their insecticidal activities. Biosurfactants may affect insect cuticles because they have an amphiphilic nature, containing both hydrophobic and hydrophilic groups. Biosurfactants may be easily sprayed onto target crops without additional additives because they are mostly soluble in water, thereby avoiding the use of synthetic organic chemicals such as solvents and other emulsifying agents. Thus, searching more environmentally friendly agents as biosurfactants has become one of the interesting topics in agricultural research programs.

The objective of the present study is to identify an insecticidal metabolite produced by microbial isolate and examine the metabolite against green peach aphid *Myzus persicae*. For this, microorganisms capable of growth on diesel oil and producing biosurfactant were isolated, and an isolate with the strongest aphid mortality was subjected to investigate insecticidal metabolites by bioassay-guided fractioning and instrumental analyses. The isolated metabolite was characterized and further examined for insecticidal activity assays under greenhouse conditions. To our knowledge, this is the first report that orfamide A is a bacterial product with insecticidal activity against aphid, *Myzus persicae*.

MATERIALS AND METHODS

Isolation of Microorganisms with Insecticidal Activity. Microorganisms were isolated by enrichment culture techniques using an agricultural soil that has the history of diesel oil contamination. Two grams of the soil samples was suspended in 100 mL of mineral salt medium (MSM) containing 1.0% (v/v) diesel oil as a sole source of carbon and the following constituents (in grams per liter, pH 7.2): K_2HPO_4,

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7.5; KH_2PO_4, 2.0; NH_4NO_3 1.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02. The suspension was incubated at 25 °C for 4 days on a shaking incubator at 150 rpm and transferred to a fresh MSM. After 5 transfers weekly, the cultures were diluted serially with the MSM and plated onto MSM agar plates that had been uniformly plated with 100 μL of diesel oil. Following microbial growth on the plates, visible colonies were obtained and purified by respectively plating on Luria−Bertani (LB, Difco) agar. Individual colonies were further tested for the oil degradation by incubating in 100 mL of the MSM containing 1% diesel oil as the sole carbon source, as described above. Isolate was identified on the basis of 16S rRNA sequence analyses, as described previously.[10] Briefly, cells grown on LB medium were harvested and digested to prepare genomic DNA using a QIAamp DNA Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Approximately 1400 base segment of 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG) and 1492r (5′-TACGGYTACCTTGTTACGACTT). PCR products with the amplified 16S rRNA gene fragment were cloned into a Promega pGEM T Easy vector. The cloned vector was transformed into *Escherichia coli* DH5α, and plasmids were obtained by using a Wizard Plus Miniprep DNA Purification Kit for nucleotide sequencing. The sequencing was carried out by a model ABI3700 Automated DNA Sequencer. Computer analysis for 16S rRNA sequence was performed with DDBJ software packages. The 16S rRNA sequence of the isolate was compared to other 16S rRNA sequences available on BLAST search of the DDBJ database. For morphological study of the isolate, a Jeol model JEM-1400 transmission electron microscopy (TEM) was employed. For screening of microorganisms with aphicidal activity, the isolates were grown in LB medium for 24 h and inoculated at 1.0% (v/v) to MSM containing 1.0% (w/v) carbon sources. The carbon sources tested were glucose, fructose, sucrose, and sodium acetate. The cultures were then incubated for 4 days and centrifuged at 12 000*g* for 20 min. The resulting supernatants were used for mortality bioassays against *Myzus persicae*.

Mortality Bioassays. Laboratory bioassays were performed using 2-week-old Chinese cabbages (*Brassica rapa*, subspecies *pekinensis* and *chinensis*). Chinese cabbage seeds disinfected with 70% ethanol were planted in commercial compost in plastic insect-breeding dishes (80 × 80 × 100 mm), and grown under greenhouse conditions for 2 weeks, as described previously.[11] The cabbage leaves were cut into small pieces (30 × 30 mm), and placed on the plates (i.d. 90 mm) layered with No. 6 filter papers that had been wet with sterilized distilled water. Twenty second-instar nymphs were reared on the cabbage leaves and allowed to settle onto the leaves for 18 h prior to application. The aphids were topically treated with 1.0 μL of the cultural supernatant obtained from above. The MSM or 10% (v/v) aqueous solvent mixture of dimethyl sulfoxide and methanol (4:1, v/v) was used as negative control samples, and imidacloprid at a recommended level (50 μg/mL) in the solvent mixture was used as positive control samples. The plates were then held in the growth chamber at 25 ± 2 °C, 65 ± 5% relative humidity, and a photoperiod of 16:8 h L:D. Aphid mortalities were investigated 24 h after topical applications by Abbott's formula as mortality (%) = $[(A - B)/A] \times 100$, where A and B represent the number of live aphids in the control samples and the number of live aphids in the treated samples, respectively.[12] All experiments were repeated in triplicate, unless otherwise stated.

Greenhouse bioassays were performed on green peach aphids, occurring naturally on greenhouse pepper plants at Nampyung (Naju, South Korea), August 2012. Seven pepper plants with high aphid populations on five leaves per plant were sprayed uniformly with the cultural supernatant to runoff using small hand-held sprayers. The plants were also received only the MSM for control samples. Temperature and relative humidity were about 28 °C and 60%, respectively, during the experiments. Aphid mortalities were investigated 24 h after spray applications as follows: mortality (%) = [(number of dead aphids after treatment)/(total number of dead and live aphids after treatment)] × 100.

Extraction and Isolation Insecticidal Metabolites. An isolate that showed the strongest aphicidal activity among the isolates was grown in the MSM (20 L) containing 1.0% (w/v) sodium acetate as the sole carbon source, as described above. The cultures were centrifuged at 12 000*g* for 20 min, and the supernatants were extracted twice with the same volume of ethyl acetate. The organic extracts (2.0 g) were evaporated to dryness in an EYELA model N-1000 rotary evaporator (Tokyo Rikakikai Co., Ltd., Japan) at 50 °C, and the extracts in chloroform were subjected to silica gel column chromatography. The column (20 mm i.d. × 50 cm in length) was eluted with 2 times the bed volume of a solvent mixture of chloroform and ethyl acetate, in which the ethyl acetate concentration was increased at 10% (v/v) in each elution step. The column was eluted again with 2 times the bed volume of a solvent mixture of ethyl acetate and methanol, in which the methanol concentration was increased at 10% (v/v) in each elution step. Each eluate was evaporated to dryness in the evaporator at 50 °C, and the extracts were dissolved at a concentration of 100 μg/mL in 0.1 M $NaHCO_3$ for aphid mortality bioassays. The eluates (900 mg) of the solvent mixture of ethyl acetate and methanol with aphicidal activity were combined and further purified by silica gel column chromatography (5 mm i.d. × 140 cm in length). The column was eluted with 4 times the volume of a solvent mixture of ethyl acetate and methanol (8:2, v/v), in which every 5 mL fraction was collected. Each fraction was then evaporated, and a part of each fraction was used for mortality bioassays as described above. The fractions (170 mg) with the aphicidal activity were combined in methanol and injected onto a Dionex model P680 model prep-HPLC equipped with a Dionex model PDA-100 photodiode array detector at 210, 254, 270, and 380 nm. The column was a μ-Bondapak C18 stainless steel column (7.8 mm i.d. × 30 cm in length). The mobile phase consisted of 60% (v/v) aqueous methanol at a flow rate of 2.0 mL/min. Peaks were collected and subjected to insecticidal bioassays, as described above. The active fraction (10 mg) with aphicidal activity was subjected to mass spectrometer (MS) and nuclear magnetic resonance (NMR) analyses: ESI-Q-TOF MS, m/z 1295.8463 $(M + H)^+$, 956.63, 827.78, 613.66, 500.81, $C_{64}H_{114}N_{10}O_{17}$; NMR, ^1H, and ^{13}C NMR (see Table 3). For determination of fatty acids of the isolated metabolite, the metabolite (2 mg) was dissolved in 2 mL of 6 N HCl solution and hydrolyzed at 110 °C in oil bath for 24 h. The solution was then

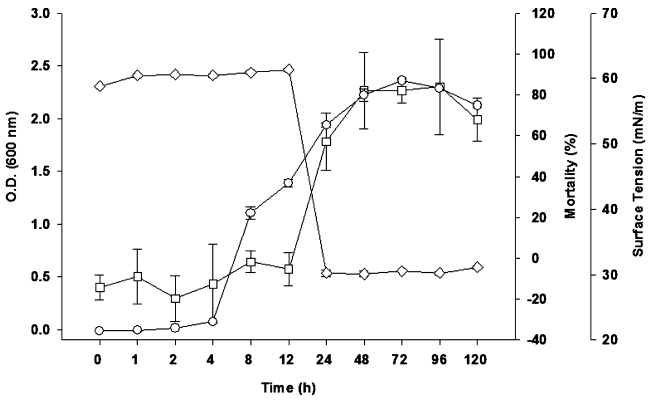

Figure 1. Time-course cell density values (○), aphid mortalities (□), and surface tension values (◇) of *Pseudomonas protegens* F6 (F6) during growth. The surface tension values and aphid mortalities were measured with cell-free supernatants of F6. The data are means ± SD of triplicate.

Table 1. Laboratory Bioassays against Aphids of Cell-Free Supernatants of *Pseudomonas protegens* F6 Grown on Sodium Acetate-Mineral Medium

fraction of cultural supernatant	aphid mortality (%)[a]
control	0.00 ± 0.00
1	81.18 ± 7.40
1/2	70.38 ± 10.19
1/5	43.26 ± 16.42

[a]The data are means ± SD of triplicate.

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extracted twice with 2 times the volume of a solvent mixture of chloroform and methanol (2:1, v/v). The organic phase was collected and dried under nitrogen gas stream. The dried extract was esterified with 10 mL of a mixture of 3 N HCl solution and methanol (7:3, v/v) at 80 °C for 1 h and extracted twice with 2 times the volume of a solvent mixture of *n*-hexane and methyl *tert*-butyl ether (1:1, v/v). The organic phase was collected and dried under nitrogen gas stream for instrumental analysis.

Table 2. Greenhouse Bioassays against Aphids of Cell-Free Supernatants of *Pseudomonas protegens* F6 Grown on Sodium Acetate-Mineral Medium

plant	aphid mortality (%)[a]
control	0.00 ± 0.00
1	69.01 ± 14.84
2	77.64 ± 23.27
3	67.50 ± 10.83
4	75.51 ± 12.24
5	51.76 ± 18.37
6	82.14 ± 22.30
7	72.14 ± 11.95

[a]The data are means ± SD of five leaves per plant.

Biosurfactant Activity Assay. Biosurfactant activity was investigated by measuring the surface tension value of water using a Du Nouy model 3010 tensiometer (Du Nouy, Japan). For the surfactant activity assay of the isolated metabolite, the metabolite was dissolved in 0.1 M $NaHCO_3$ and subjected to examine surface tension values after serially diluted. Biosurfactant measurements were also performed on cell-free supernatants obtained as described above. The instrument was calibrated with air and water to a reading of 72.75 mN/m. All measurements were performed triplicate, unless otherwise stated.

Instrumental Analyses. Mass spectra were obtained using a Bruker Daltonics time-of-flight mass spectrometry (TOF MS) connected with a Dionex model P680 high performance liquid chromatography (HPLC) equipped with a Dionex model PDA-100 photodiode array detector. MS analyzer was calibrated with a sodium formate solution consisting of a mixture of 10 mM sodium hydroxide in isopropanol and 0.2% (v/v) formic acid at a ratio of 1:1 (v/v), as described previously.[13] For the detection of fatty acids of the isolated metabolite, a Shimazu model QP2010 gas chromatography mass spectrometry (GC−MS) system equipped with a DB-5 capillary column (0.25 mm i.d. × 30 m in length, 1.0 μm film thickness) was employed in electron impact (EI) and chemical ionization (CI) modes. The EI energy was 70 eV, and the CI gas was isobutane. Nuclear magnetic resonance (NMR) spectrometers were a Varian model Unity INFINITY plus 200 NMR (200 mHZ) for ^1H analysis

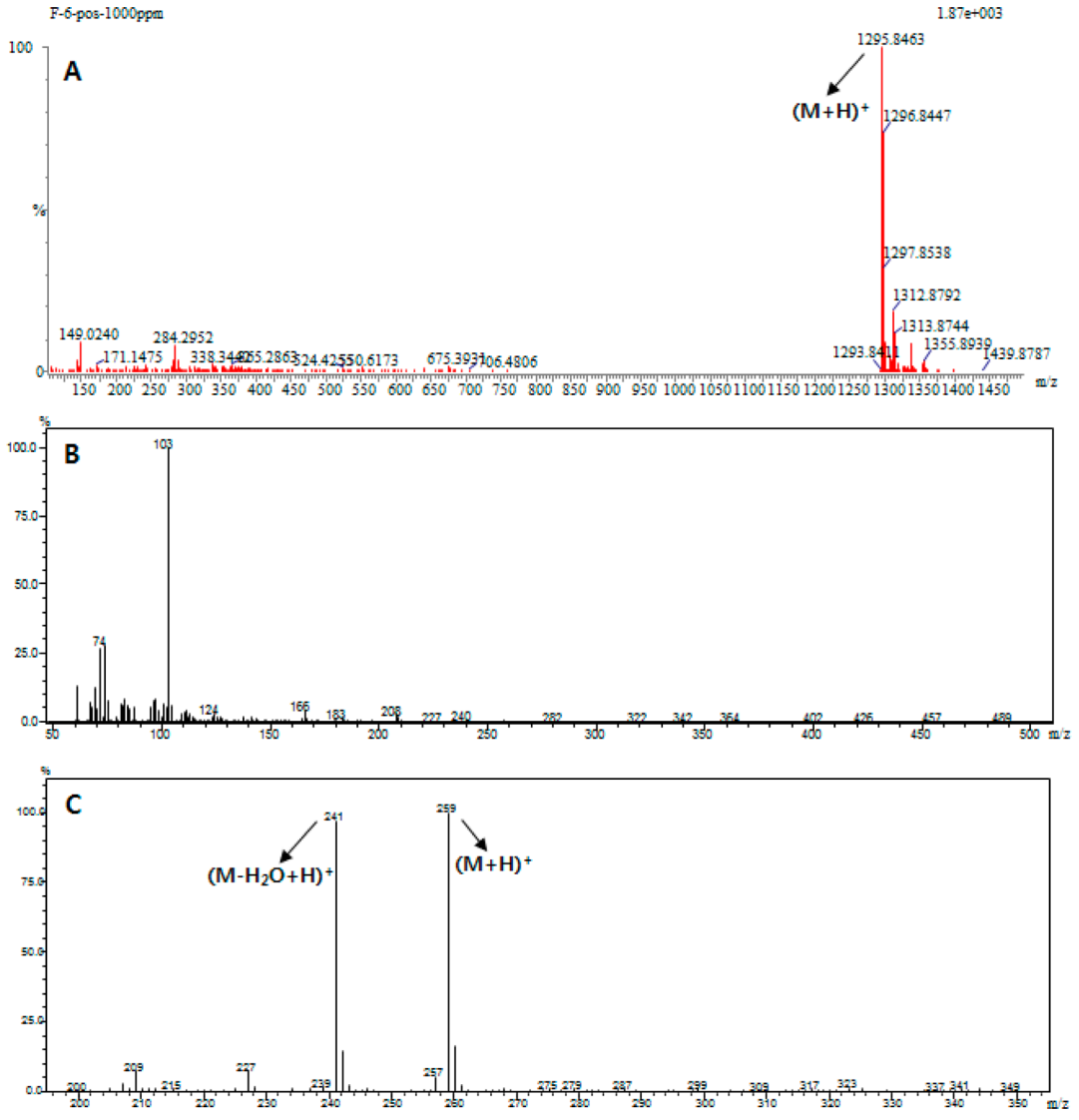

Figure 2. TOF MS spectrum for the isolated orfamide A (A) and GC−MS spectra in the EI (B) and CI (C) modes of methyl-esterified 3-hydroxytetradecanoic acid (3-OH-TDA) produced by acid hydrolysis of the metabolite orfamide A.

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Table 3. ^{1}H and ^{13}C NMR Data of Orfamide A Isolated from Cell-Free Supernatants of *Pseudomonas protegens* F6

name	position	δ_H	δ_C	name	position	δ_H	δ_C
L-Leu1	1	4.09	53.8		33	1.75	25.5
	2	1.68	40.5		34	0.92	21.5
	3	1.69	25.6		35	0.99	23.3
	4	0.95	22.4		36		175.2
	5	0.99	23.3	L-Leu4	37	4.31	54.1
	6		175.8		38	1.74	41.1
D-Glu	7	4.09	57.1		39	1.72	25.6
	8	2.02	27.8		40	0.93	21.6
	9	2.35	34.5		41	0.98	23.3
	10		nd[a]		42		175.3
	11		nd	D-Ser2	43	4.35	57.3
D-*allo* Thr	12	4.34	62.8		44	3.82	62.5
	13	5.37	70.6		45		172.5
	14	1.38	18.4	L-Val	46	4.36	59.5
	15		nd		47	2.15	31.1
D-*allo* Ile	16	3.89	62.5		48	0.85	18.9
	17	2.03	36.7		49	0.90	19.6
	18	1.19, 1.48	26.6		50		171.1
	19	0.93	11.4	3-OH-TDA	1′		175.1
	20	0.99	16.1		2′	2.40	44.2
	21		nd		3′	4.08	69.6
L-Leu2	22	4.19	54.7		4′	1.51	38.4
	23	1.57, 1.79	40.5		5′	1.30	30.4
	24	1.79	25.3		6′	1.30	30.4
	25	0.87	20.8		7′	1.30	30.4
	26	0.92	23.3		8′	1.30	30.4
	27		175.6		9′	1.30	30.4
D-Ser1	28	4.37	57.8		10′	1.30	30.4
	29	3.84, 3.96	62.5		11′	1.30	30.4
	30		172.8		12′	1.30	30.4
L-Leu3	31	4.44	53.4		13′	1.30	30.4
	32	1.52, 1.80	41.3		14′	0.91	14.0

[a]Not detected.

and a Varian model Unity INFINITY plus 600 NMR (600 mHz) for ^{13}C analysis. For NMR analyses, the sample was prepared in CD_3OD.

Statistical Analysis. Data were analyzed by Analysis of Variance (ANOVA) using IBM SPSS Statistics 19 software (Somers, NY). The concentration-dependent mortality data were obtained by probit analysis (95% confidence limits) using SAS program.

RESULTS AND DISCUSSION

Microorganisms capable of growth on diesel oil were of interest in this study because they are known to produce variable biosurfactants.[14−16] An isolate that showed the best growth on diesel oil was examined for further study. The 16S rRNA sequence analysis of the isolate showed 100% similarity with *Pseudomonas protegens* CHA0^T with accession number AJ278812 (see the Supporting Information), a bacterium that produces the compound 2,4-diacetylphloroglucinol, which shows biocontrol activity against plant pathogens.[17] TEM analysis of the isolate showed a rod-shaped bacterium with polar flagella. On the basis of the data, the isolate was designated *Pseudomonas protegens* F6 (F6). Sequences obtained from F6 were deposited in the GenBank database under accession number JQ836664.

Aphid mortalities accompanied F6 during growth on sodium acetate MSM (Figure 1), producing the highest mortality after 48 h of incubation. The supernatants of F6 grow on sodium acetate MSM for 96 h showed about 81% of aphid mortality, producing about 70.38% and 43.26% mortalities after being diluted 2 and 5 times in the MSM, respectively (Table 1). The growth of F6 was also accompanied by a decrease in the surface tension value to about 30.0 mN/m when incubated in sodium acetate MSM for 96 h (Figure 1), suggesting that F6 produced a biosurfactant during growth. The growth-dependent data suggested that insecticidal metabolites produced by F6 were related to biosurfactant production. Greenhouse experiments of the cultural supernatants of F6 showed aphid mortality ranging from about 51.76% to 82.14% (Table 2). The supernatant treatment did not cause phytotoxic symptom on the pepper plants, as judged by visual observation. Thus, the data suggested that the cultural supernatant of F6 could be used for aphid control.

To identify the insecticidal metabolite produced by F6, the cultural extracts were subjected to chromatography and instrumental analyses. TOF MS analysis of the isolated metabolite detected $(M + H)^+$ peaks at m/z 1295.8463 (Figure 2A) and afforded the molecular formula $C_{64}H_{114}N_{10}O_{17}$. When the metabolite was acid hydrolyzed and methyl-esterified, a methylated fatty acid was detected by GC/MS analyses in the electron impact (EI) and chemical ionization (CI) modes (Figure 2B and C). GC−MS in the EI mode detected a main fragment ion peak at m/z 103, which was identified as 3-hydroxy-tetradecanoic acid (3-OH-TDA) on the basis of a library search. GC−MS in the CI mode detected a $(M + H)^+$ peak at 259 and a $(M + H - H_2O)^+$ peak at 241, identical to the MS patterns of 3-OH-TDA. MS/MS analyses of the isolated

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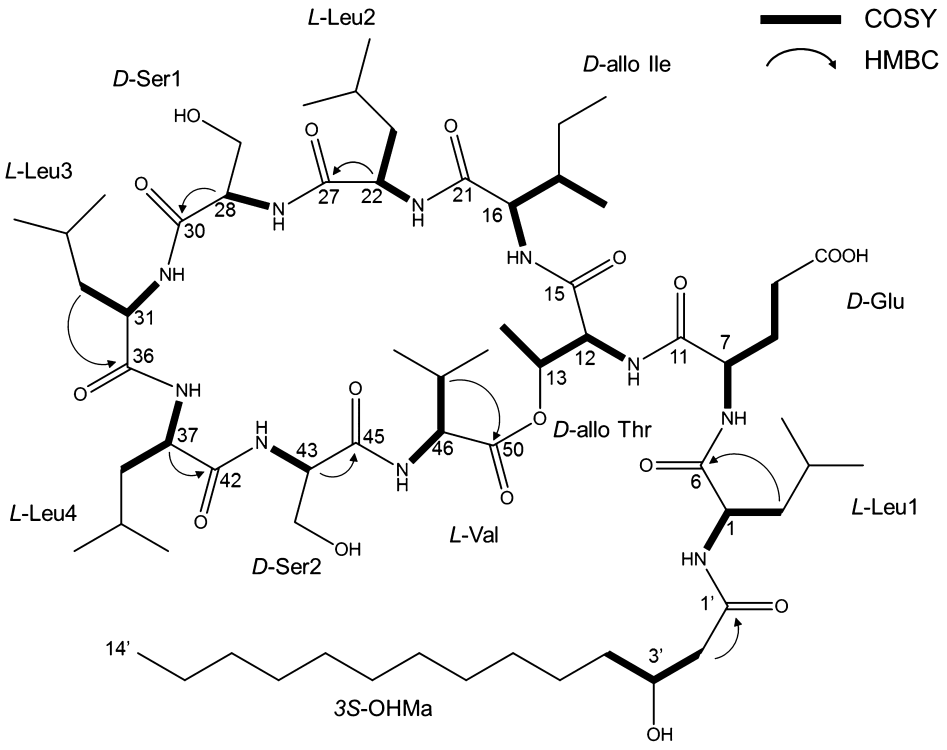

Figure 3. HMBC and COSY correlations of orfamide A isolated from cell-free supernatants of *Pseudomonas protegens* F6.

metabolite produced *m/z* 956.63, 827.78, 613.66, and 500.81 as the main fragmentation ions, giving subsequence losses of the ions 3-OH-TDA-leucine1, glutamic acid, threonine-isoleucine, and leucine2, and leaving serine1-leucine3-leucine4-serine2-valine as the final fragment ion.

The presence of amino acids and 3-OH-TDA in the isolated metabolite was further confirmed by NMR analyses. The ^{1}H and ^{13}C chemical shift data of the isolated metabolite are presented in Table 3. The NMR spectra exhibited the typical resonances of peptides: α-proton resonances between δ 3.89 and 4.37, side-chain protons at δ 0.85−2.02, and carbonyl resonances at δ 171.1−175.8. Multiple NMR experiments, including $^{1}H-^{1}H$ gCOSY, $^{1}H-^{13}C$ gHMBC, and $^{1}H-^{13}C$ gHSQC, were performed to identify the molecular structure of the metabolite. Three carbonyl carbons out of 12 were not detected in the HMBC spectrum. The NMR and MS data of the isolated metabolite were identical to those of orfamide A, previously reported to be produced by other the *Pseudomonas* strain.[18] Thus, the molecular formula of the isolated metabolite was deduced as $C_{64}H_{114}N_{10}O_{17}$, consisting of 10 cyclic amino acids and a long fatty acid fragment as 3-OH-TDA (Figure 3), which was identical to the formula afforded by TOF MS.

A dose-dependent mortality was observed for aphids treated with the isolated orfamide A (Figure 4), suggesting that the metabolite clearly affected aphids. The LC_{50} value of the isolated orfamide A was estimated to be 34.5 μg/mL (95% confidence limits) by probit analysis. The isolated orfamide A showed more than 80% mortality at 100 μg/mL. Imidacloprid, a commercial insecticide as a positive control sample, showed 100% mortality at a recommended dose level (50 μg/mL), while the solvent mixture and mineral salt medium as negative control samples showed less than 10% mortality. These suggest that our bioassay system was satisfactory. The metabolite orfamide A was subjected to a biosurfactant activity assay to investigate whether the biosurfactant activity of cell-free supernatants of F6

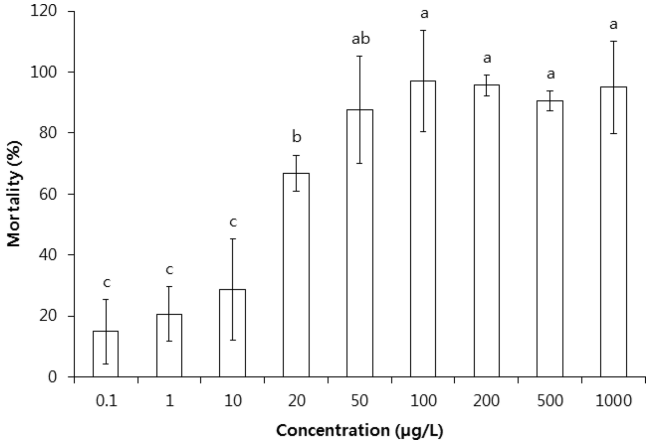

Figure 4. Dose-dependent aphid mortalities of orfamide A isolated from cell-free supernatants of *Pseudomonas protegens* F6. The data are means ± SD of triplicate. The data with the same letter in the same column did not differ significantly ($P < 0.05$).

was related to the metabolite. Treatment with orfamide A at 10 μg/mL caused a considerable decrease in the surface tension value of water to about 37.5 mN/m (Figure 5), suggesting that the growth-dependent aphicidal and biosurfactant activities in the cell-free supernatant (Figure 1) were due to the orfamide A.

Biosurfactants derived from microorganisms have been gaining increased attention and interest in developing environmentally friendly, safe, and integrated pest management programs.[19,20] Few biosurfactants for aphid control have been reported. In the present study, bioassay-guided isolation and instrumental analyses of cell-free supernatants of F6 identified orfamide A as a major aphicidal metabolite. Laboratory and greenhouse mortality bioassays suggested that orfamide A may be applicable to control aphids in vitro and in vivo. Orfamide A was suggested to induce dehydration of the cuticle of aphids because it is a surface-active

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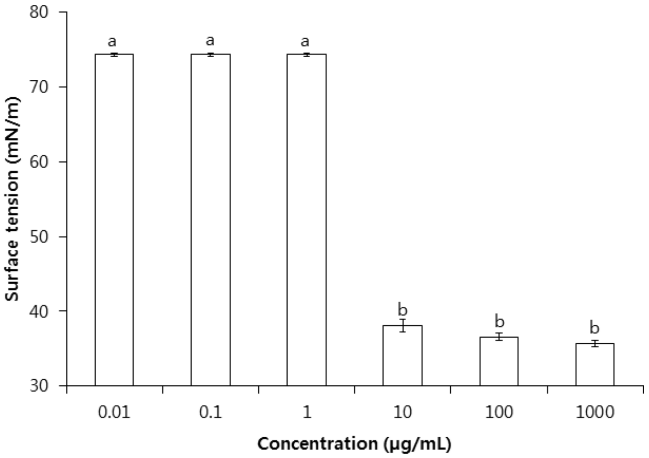

Figure 5. Water surface tension values of orfamide A isolated from cell-free supernatants of *Pseudomonas protegens* F6 grown on sodium acetate-mineral salt medium for 96 h. The data are means ± SD of triplicate. The data with the same letter in the same column did not differ significantly ($P < 0.05$).

compound that interacts with cuticle molecules such as fatty acids and phospholipids, as was previously demonstrated by other surface-active compounds.[21] Aphid membrane disruption caused by biosurfactant has been previously reported by other biosurfactant rhamnolipid.[22] Rhamnolipid induced significant dehydration of the cuticle membrane of aphids to cause their death. The effects of the orfamide A on aphids may be similar to those of rhamnolipid, both of which are amphiphilic compounds with surface-active properties. The cuticle membranes of aphid consist of variable mixtures such as alkanes, wax esters, fatty acids, and phospholipids. These are lipophilic molecules that would interact with biosurfactant with both a lipophilic and a hydrophilic moiety. The biosurfactant properties of the metabolite orfamide A were suggested to affect cuticle lipids and lead to membrane perturbation. This hypothesis was supported by the microscopy observations that showed significant changes in the endocuticle layer of aphids by orfamide A treatment (see Supporting Information). Taken together with previous work, biosurfactants hold potential for use as an environmentally friendly agent to control aphids.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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