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Characterization of Antimicrobial Compounds Produced by *Pseudomonas aurantiaca* S-1

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Abstract

Pseudomonas aurantiaca S-1 can serve as a natural source of pesticides towards phytopathogens like *Fusarium oxysporum* P1 and *Pseudomonas syringae* pv. *glycinea* BIM B-280. The largest pool of produced antimicrobial compounds was found in four days-old spent culture supernatant. At least two groups of bioactive substances were identified, one responsible for the antibacterial activity and the other for the antifungal activity. The fraction with strong antibacterial activity had the molecular mass 282.8 and formula $C_{18}H_{36}NO$, and the fraction with strong antifungal activity had molecular mass 319.3 and molecular formula $C_{20}H_{31}O_3$ which could be a new fungicide. Additionally, *P. aurantiaca* S-1 was able to produce indoleacetic acid and siderophores.

Key words: *P. aurantiaca* S-1, antimicrobial compound, bioautography, indoleacetic acid, siderophore

Introduction

Interest in replacement of synthetic chemicals by biological alternatives is a current trend in the area of combating plant pathogenesis. Synthetic chemicals can negatively affect the environment, human and animal health (Hodgson and Levi, 1996; Zwir-Ferenc and Biziuk, 2004). An alternative to the synthetic compounds are natural, environmentally friendly pesticides (biopesticides) (Boland and Kuykendall, 1998). One of the major sources of biopesticides are microorganisms.

One of the genera having the potential to produce bioactive compounds against plant pathogens is *Pseudomonas*. These genera are also known for the production of metabolites which stimulate plants growth and colonization of plant roots (Boland and Kuykendall, 1998; Mikuriya *et al.*, 2001; Kang *et al.*, 2006). A wide spectrum of antimicrobial components towards phytopathogenic fungi and bacteria are produced (Leisinger and Margrafft, 1979; Chin-A-Woeng *et al.*, 2003; Cazorla *et al.*, 2006). Pseudomonades usually produces several metabolites from different groups such as indoles (Mikuriya *et al.*, 2001; Kang e*t al.*, 2006), phenazines (Chin-A-Woeng *et al.*, 2003; Kumar e*t al.*, 2005; Cazorla *et al.,* 2006), pyocyanine, pyrrolnitrin (Ligon *et al.*, 2000), pyoluteorin, acetylphloroglucinols (Raaijmakers *et al.*, 1999; Guihen *et al.*, 2004), tenzin (Nielsen *et al.*, 2000), pseudotrienic acids A and B (Pohanka *et al.*, 2005) and viscosinamide (Nielsen *et al.*, 1998). *P. aurantiaca* can also synthesize antimicrobial compounds having the same structure as produced by other members of pseudomonades: phenazines (Feklistova and Maksimova, 2005), proteins (Kang *et al.*, 2004) and phloroglucinols (Pidoplichko and Garagulya, 1974). Mycolytin is an antifungal biopesticid formed by *P. aurantiaca* M-518 (Omel'yanets and Mel'nik, 1987).

To elucidate the mechanism of biopesticides action and to upgrade the quantity and quality of their synthesis, it is important to identify the bioactive components structure.

In this paper some new antimicrobial compounds, indoleacetic acid and siderophores from *P. aurantiaca* S-1 are described.

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Experimental

Materials and Methods

Microbial strains and growth media. *Pseudomonas aurantiaca* S-1 strain was isolated from municipal sludge containing cellulose and lignin in the Laboratory of Biological Control, Institute of Microbiology National Academy of Science, Minsk, Belarus. The strain was cultivated in brain heart broth (BHB, Merck) in 100 ml liquid media (pH 7-7.2) on the rotary shaker (120 rpm) and harvested after 96 h at 28°C.

Phytopathogenic cultures of *Fusarium oxysporum* P1, and *Pseudomonas syringae* pv. *glycinea* BIM B-280 were from the collection at the Institute of Microbiology National Academy of Sciences, Belarus (BIM) and were used to check the antimicrobial properties of *P. aurantiaca* S-1.

The plant pathogenic fungus *F. oxysporum* P1 was cultivated on potato dextrose media (pH 6) and the pathogenic bacteria *P. syringae* pv. *glycinea* BIM B-280 in the Luria-Bertani Media (LB), (g/l): Bacto Tryptone (10.0), Yeast Extract (5.0), NaCl (5.0), pH 7.2.

Antimicrobial activity assay. The measurement of antifungal and antibacterial activity was carried out using the wells method (Segi, 1983). Test agar plates were prepared with a bottom layer of 15 ml LB solidified with agar (Difco Agar, 2%) which was overlayed with 4 ml of semi-solid LB (Difca Agar 1.2%) mixed with 1 ml of a phytopathogenic microorganism (harvested at stationary phase). On each agar plate, wells of 9 mm in diameter were made and *P. aurantiaca* S-1 was added to each well.

Zones of pathogen growth inhibition were measured after 24 h (bacteria) and 48 h (fungus) incubation at 24–28°C. The diameter of zone inhibition minus the well diameter was the measure of antimicrobial activity. Wells filled with sterile growth media, served as negative control.

Presence of antimicrobial compounds and the effect of heat treatment assay. Antimicrobial activity was tested using Spent Culture Supernatant (SCS) from four days old culture, after centrifugation at 10 000 × g for 15 min, 4°C. The separated cells were suspended in water, immersed in ice and then sonicated using a standard Microtip (Sonicator™ Ultrasonic Liquid Processor, New York). The setting was: cycle time 5 sec, 20% of duty cycle and 5% output limit. Each sample was sonicated for 5 min with 1 min intervals. All the samples were separately tested for the presence of antimicrobial compounds against *F. oxysporum* P1 and *P. syringae* pv. *glycinea* BIM B-280 in an antimicrobial assay of the wells methods (Segi, 1983). To test the effect of heating; the SCS samples were heated in a boiling water bath for 15 min, and then cooled by immersing the sample in an ice bath.

Fractionation of antimicrobial compounds using C18 solid phase extraction (SPE). Sample treatment: 5 ml of SCS was adjusted to pH 2.0 with 0.1% trifluoroacetic acid (TFA) and passed through a SPE disposable column containing C18 (500 mg) (Varian, Inter/Analytical Industry). The column was pre-equilibrated with 2.5 ml of 10% acetonitril (CH_3CN) in 0.1% TFA water solution. Fractionation was carried in two different ways. In the first method, a step-gradient was used, from 20% to 100% CH_3CN in 0.1% TFA water solution, using 2.5 ml portions of each mixture. The steps were: 20, 30, 40, 50, 60 and 70% of CH_3CN. Each fraction was tested by bioautography (against phytopatogenic fungal and bacteria). Based on the bioautography results, the elution pattern was changed. In the second approach the elution process started with 50% CH_3CN followed by 80, 90 and 100% CH_3CN. After removal of CH_3CN the samples were tested by bioautography. Additionally, for each bioactive fraction, the ultraviolet-visible spectra were determined using a Beckman Coulter DU 800 spectrophotometer and Mass spectra – a Q-TOF Micro (Micromass®) Mass spectrometer.

Bioautography. Silica gel 60 on thin layer chromatography (TLC) aluminum foil (Merck) was cut into small strips and 100 µl size samples were applied and dried. The tested samples were Spent Culture Supernatant (SCS) before chromatography and fractions were eluted with different concentration of acetonitril from C18 SPE column. An agar layer (15 ml with 2% LB) was prepared. On top of the layer was placed a piece of TLC aluminum foil with the sample to be analyzed. It was then covered with top agar with 1.2% LB (4 ml) and 1 ml of phytopathogenic microorganism culture supernatant (harvested at stationary phase). The plates were then incubated for 24 h at 24°C (bacteria) or for 48 h at 28°C (fungus). A semiquantitative antimicrobial activity was determined by the degree of inhibition of pathogen growth. This bactericidal effect was visualized by using methyl thiazolyl tetrazolium (2.5 mg/ml), which was converted to an intense blue color by the dehydrogenases of living microorganisms (Hamburger *et al.*, 1987).

Mass-spectrometric (MS) analyze of antimicrobial compounds. After SPE chromatography the bioactive fractions were analyzed on a Q-TOF Micro (Micromass®) Mass spectrometer equipped with an electrospray ionization source operated in positive ion mode. The positive electrospray ionization conditions included a capillary voltage of 2.31 kV, a cone voltage of 40 V, ion energy of 4 V, desolvation temperature of 120°C and source temperature of 80°C. Samples were introduced using a syringe pump with flow rates of 10 ml/min. 2-Phenazinol, 2-phenazinecarboxylic acid found in culture filtrates of pseudomonades standards were

used as purchased (Aldrich). Data were analyzed by MassLynx 4.0 SP1.

Detection of indoleacetic acid. Reagent for the detection of indoleacetic acid was prepared from 1.0 ml of 0.5 M $FeCL_3$ and 50 ml 35% $HCLO_4$. Two ml of reagent was added to 1 ml of SCS and stirred for 25 min. The mixture was centrifuged at 6000×g for 10 min, 4°C and at 530 nm the absorbance was measured in the supernatant (Gordon and Weber, 1951).

Determination of siderophores formation. One ml of SCS was mixed with 40 µl of 1 M $FeCL_3 \times 6H_2O$ (Neilands, 1995). UV-VIS spectra were determined and compared to the spectra of the growth medium treated similarly.

Results

Localization of antimicrobial compounds. Table I shows the bioactivity measurement for metabolites in culture broth, SCS, homogenized cells and heat-treated SCS. The antimicrobial activity found in SCS was almost as high as the activity present in the culture broth. The major part of the activity was found in the SCS. The bioactivity found in the cells corresponded to about 30–36% of the activity found in the culture broth. In the heat treated SCS the same antimicrobial activity was found as in the untreated SCS.

Indoleacetic acid and siderophores. The samples of SCS showed positive qualitative tests for indoleacetic acid and siderophores. SCS after specific reaction on the indoleacetic acid showed red color, and dark brown on the siderophores (data not shown).

Fractionation of antimicrobial compounds by using C18 SPE column. In Table II is shown that the biological activity began eluting with 50% of CH_3CN with maximal amount at 100% of CH_3CN. In Table III another elution pattern is shown, where a strong antifungal activity was eluted with 80% of CH_3CN whereas antibacterial activity was eluted with 90% of CH_3CN.

Table I
Localization of antimicrobial metabolites in SCS and cells from stationary phase of *P. aurantiaca* S-1. The effect of heat treatment on SCS

Phytopathogen	Antimicrobial activity* (mm)			
	Culture broth	SCS	Homo-genated cells	Heat treated SCS
P. syringae pv. *glycinea* BIM B-280	12 ± 0.9	10±0.6	3±1.0	9±0.6
F.oxysporum P1	13±0.5	11±0.3	4±0.9	11±0.9

* Diameter of growth inhibition by the wells method see "Materials and Methods"). Triplicate measurement for each value.

Table II
Bioautography of fraction eluted from C18 solid phase column*

Fraction/ elution (% of CH_3CN)	Antimicrobial activity of fraction SCS *P. aurantiaca* S-1	
	F. oxysporum P1	*P. syringae* pv. *glycinea* BIM B-280
Non adsorbed	–	–
20	–	–
30	–	–
40	–	–
50	++	++
60	++	++
70	++	+++
100	+++	+++

* Fraction from first trial – applying step gradient (20–100% CH3CN) (see "Materials and Methods").
Antimicrobial activity: (–) no zone of inhibition, (++) inhibitory, (+++) strong inhibition.

Fig. 1 shows the ultraviolet-visible spectra of fractions with antimicrobial properties. As example the fraction eluted with 80% of CH_3CN showed a peak at 270 nm, 390 and about 420 nm. The fraction eluted with 90% of CH_3CN showed a major peak at 289 nm and a very small peak at 415 nm. These results indicate that the fractions with antibacterial and antifungal activities contain different compounds.

Identification of antimicrobial metabolites by MS. Fractions shown in Table III were analyzed by MS. In Fig. 2, it is seen that the fraction eluted with 90% CH_3CN had high intensity of m/z peak 282.3 while in Fig. 3 the fraction eluted with 80% CH_3CN had the highest intensity of m/z peak 319.3. These peaks could correspond to substances with strong antibacterial activity with molecular mass 282.3 and molecular formula $C_{18}H_{36}NO$, and the fraction with strong antifungal activity with molecular mass 319.3

Table III
Bioautography of fraction eluted from C18 solid phase column*

Fraction/ elution (% of CH_3CN)	Antimicrobial activity of fraction SCS *P. aurantiaca* S-1	
	F. oxysporum P1	*P. syringae* pv. *glycinea* BIM B-280
Non adsorbed	–	–
50	+	+
80	+++	+
90	+	+++
100	+	+

* Fraction from second trial – applying step gradient (50–100% CH3CN) (see "Materials and Methods").
Antimicrobial activity: (–) no zone of inhibition, (+) inhibitory, (+++) strong inhibition.

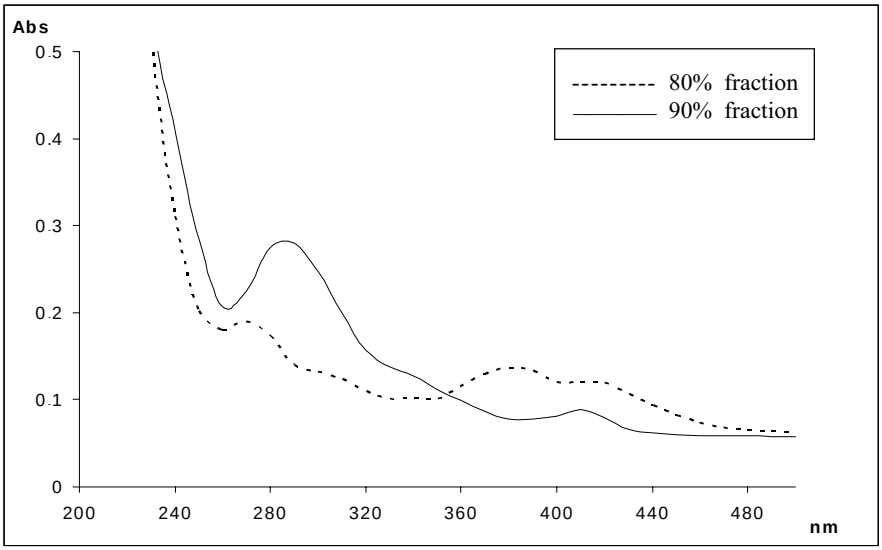

Fig. 1. Ultraviolet-visible spectra of fractions with antimicrobial properties eluted as it is shown in Table III.

and molecular formula $C_{20}H_{31}O_3$. Commercial standards of 2-phenazinol (C_{12} H_8 N_2 O) and 2 phenazinecarboxylic acid (C_{13} H_8 N_2 0_2) with different molecular weights were used.

Discussion

Many investigators have suggested the rhizospheric bacteria *Pseudomonas* spp. as very interesting sources for the identification of antimicrobial compounds and their practical use as biopesticides (Chin-A-Woeng *et al.*, 2003; Cazorla *et al.*, 2006). *Pseudomonas* spp. can be introduced into soil that abounds in natural rhizosphere habitats (Rensen *et al.*, 2001). Pseudomonades have been shown to produce a wide spectra of compounds with antimicrobial activity against phytopathogenic fungi and bacteria (Chin-A-Woeng *et al.*, 2003; Pohanka *et al.*, 2005; Cazorla *et al.*, 2006). They can also promote plants and beneficial soil microorganisms growth, synthesize phytohormones (Arshad and Frankenberger, 1991) and siderophores (Leong, 1986), convert difficult utilizable compounds to nutrients for plants and other microorganisms (Venturi *et al.*, 1998).

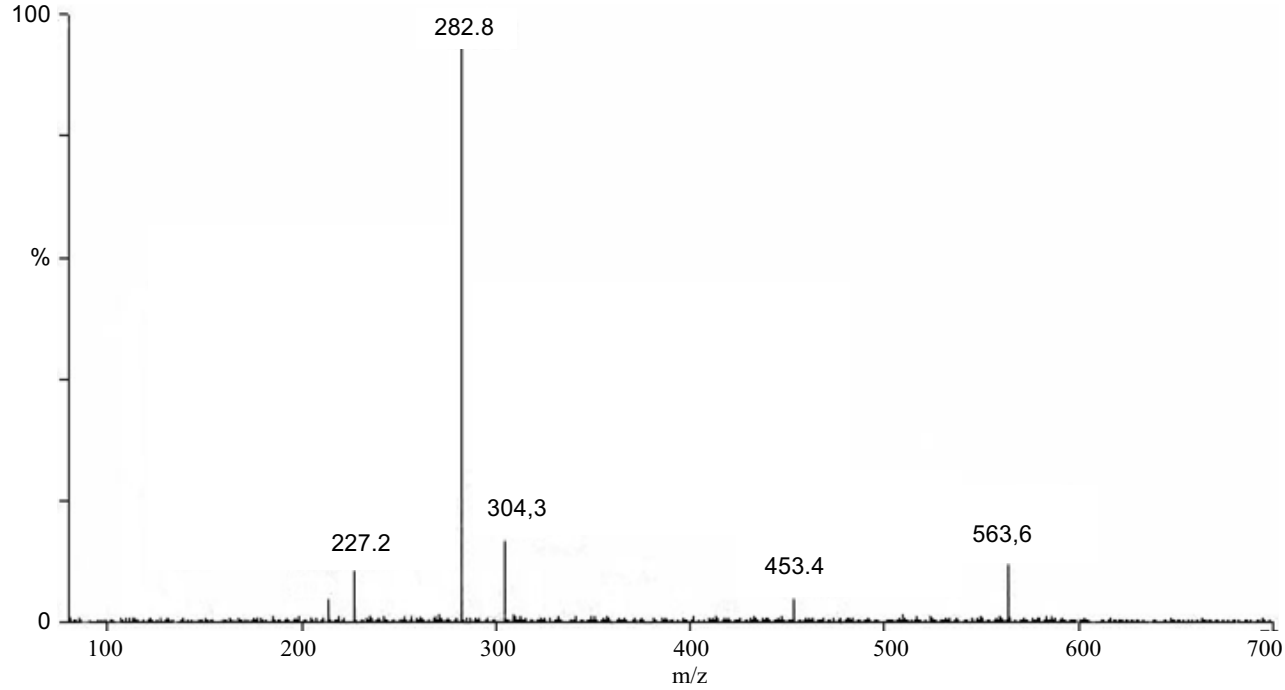

Fig. 2. Mass spectrometry of active fractions eluted with 90% CH_3CN

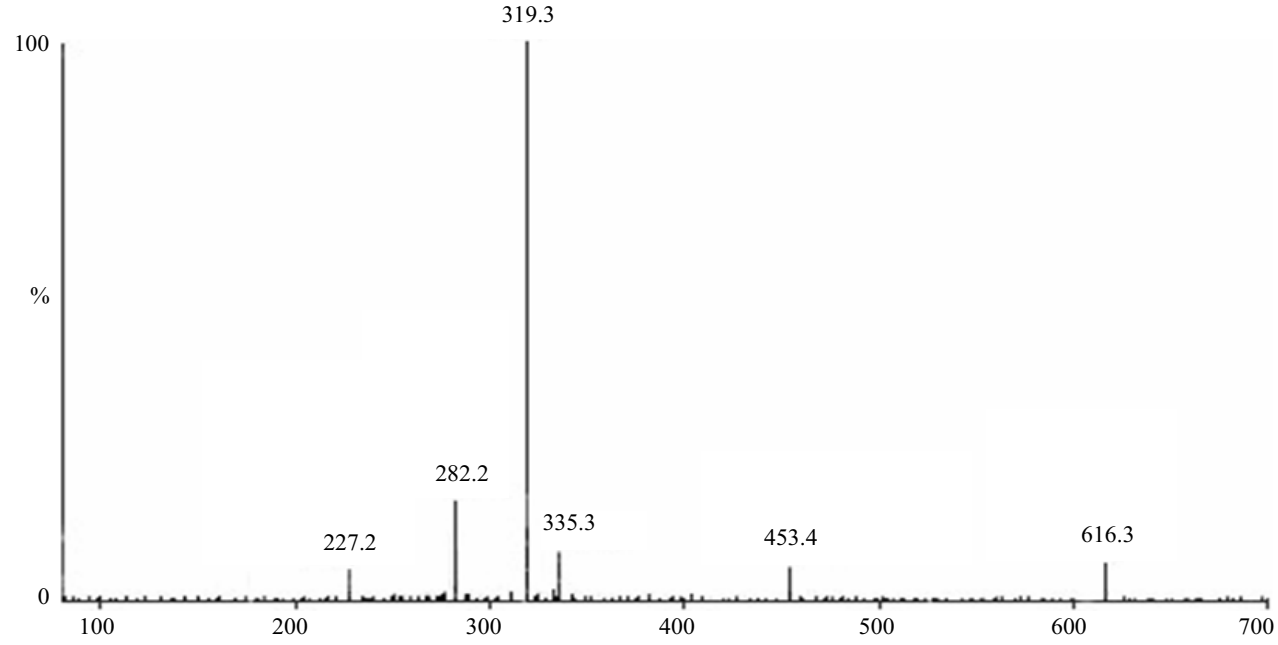

Fig. 3. Mass spectrometry of active fractions eluted with 80% CH_3CN

The investigated *P. aurantiaca* S-1 may produce indoleacetic acid-like substances (Patten and Glick, 2002) and siderophores. These can stimulate plants growth and can display antimicrobial capacity (Smirnov and Kiprianova, 1990). The ability to synthesize indoleacetic acid and siderophores may thus play an important role in the use of *P. aurantiaca* S-1 as potential biofertilizers with biopesticides function.

Many investigations have identified the structure of antimicrobial compounds from *Pseudomonas* spp. such as phenazines (Kumar *et al.*, 2005; Cazorla *et al.*, 2003; Chin-A-Woeng *et al.*, 2006), pyocianin, pyrrolnitrin (Ligon *et al.*, 2000), pyoluteorin (Nowak-Thompson *et al*., 1990), acetylphloroglucinol (Raaijmakers, 1999; Guihen *et al*., 2004), tenzin, pseudotrienic acids A and B (Pohanka *et al.*, 2005) and viscosinamide (Nielsen *et al.*, 1998). Pseudomonades strains often synthesize more than one antimicrobial compound *e.g*. different phenazines, phenazine-1-carboxylic acid and 2,4-diacetylploroglucinol, pyoluteorin and pyrrolnitrin (Paul and Sarma, 2006), monoacetylphloroglucinol and 2,4-diacetylploroglucinol (Guihen *et al.*, 2004) and other combinations. An important class of secondary metabolites is phenazines. Phenazines are heterocyclic nitrogen containing substances and show antimicrobial activity to phytopathogenic bacteria and fungi (Chin-A-Woeng *et al.*, 2003). More then 50 natural phenazines are known and some microorganisms can synthesis more than 10 phenazines derivatives (Chin-A-Woeng *et al.*, 2003). Well known phenazines occurring as metabolites in pseudomonades are phenazine-1-carbo-xylic acid, phenazine-1-carboxamide, aeruginosin A, pyocyanin, 2-hydroxyphenazine-1-carboxylic acid, 1-hydroxyphenazine (Thomashow *et al.*, 1990; Raaijmakers *et al.*, 1997; Price-Whelan *et al.*, 2006).

Single Mass analysis indicated that a substance with composition $C_{18}H_{36}NO$ was an important compound. This substance was found in the fraction eluted with 90% CH_3CN (Table III, Fig. 2). This fraction also showed different spectra in comparison to the spectra of the fraction with strong antifungal activity (Fig. 1) with the major peak at 270 nm. The spectra of antimicrobial fraction does not correspond to the phenazinol or phenazinolcarboxilic acid standards used. $C_{18}H_{36}NO$ is a cyclic aromatic N-containing substance and correspond to the new variety of pyo-compounds (could be also alkyl quinolinol) (Leisinger and Margrafft, 1979). The derivatives of the decahydroquinolin alkaloids (Michael, 2005), 8-hydroxy-4-methoxy-quinalic acid (quinolobactin) (Mossialos *et al.*, 2000) are close to the structure of $C_{18}H_{36}NO$. Substance with $C_{18}H_{36}NO$ (morpholinum) had shown fungicides property (Schlueter and Weltzien, 1971).

A strong antifungal activity of the fraction eluted with 80% CH_3CN (Fig. 3) had a molecular formula $C_{20}H_{31}O_3$. The data base search (ACS 2007) lacks reference corresponding to the found formula. Therefore, it will be interesting to purify a large amount and to make *e.g*., NMR and describe the structure of the new fungicide.

Our investigation is an important start to the study of antimicrobial secondary metabolites in *P. aurantiaca* S-1. More detailed studies of the compounds indicated by single mass detection are planned. This will lead to the precise identification of new antimicrobial components from *P. aurantiaca* S-1, important for the production of useful biopesticides.

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