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Aerugine, an Antibiotic from *Streptomyces fradiae* Strain SU-1

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ABSTRACT

The antibiotic aerugine (4-hydroxymethyl-2-(2-hydroxyphenyl)-2-thiazoline) was isolated from the culture filtrate of *Streptomyces fradiae* strain SU-1 and purified by Sephadex LH-20 and silica gel column chromatography. The structure was determined by detailed interpretation of ^{1}H, ^{13}C NMR and mass spectra. The minimum inhibitory concentrations (MIC) of aerugine were 12.5 µl/ml determined against both *Colletotrichum gloeosporioides* and *Phytophthora parasitica.* It completely inhibited conidial germination of *C. gloeosporioides* and inhibited encysted zoospore germination of *P. parasitica*.
Key words: aerugine, thiazoline, *Streptomyces fradiae, Colletotrichum gloeosporioides*, *Phytophthora parasitica*

INTRODUCTION

Fungal pathogens are important disease causal agents found in a variety of plants. The control of fungal diseases in modern agriculture is mainly achieved by an intensive use of chemical fungicides. However, concern about the environmental influences such as toxic soil and water contaminations, pesticide residues in plants and the high costs of chemicals is increasing. It encourages farmers and researchers to look for bio-control agents and resistant cultivars. Numbers of fungi and bacteria have been successfully developed as bio-control products such as *Streptomyces griseoviridis* strain K61 (Mycostop), and *Streptomyces lydicus* (Actino-Iron and Actinovate Plus/M) to control *Rhizoctonia, Pythium*, *Botrytis*, *Fusarium* and root rot diseases (Hewitt, 1998; Steven, 2002). Streptomycetes are among the richest sources for antibiotics. Several *Streptomyces* antibiotics are used in human and veterinary medicine, agriculture, and fishery industry. Many antibiotic substances were reported to be isolated from *Streptomyces fradiae* such as fradicin (Hickey and Hidy, 1951), fradicin-mycelin group substances (Igarashi *et al*., 1955), neomycin (Waksman *et al.*, 1957), mycelin, mukherjee, frenolicin (Waksman and Lechevalier, 1962), phosphonomycin (Stapley *et al.*, 1969), dekamycin (Truong *et al.*, 1977), tylosin (Baltz *et al.*, 1981), urdamycins (Drautz *et al.*, 1986), actinomycin Z (Bossi *et al.*, 1958), and actinomycin Z complex (Lackner *et al.*, 2000). The aim of this study was to purify and characterize an antifungal agent from *Streptomyces fradiae* SU-1, a selected strain from Thailand. The characterization was focused on the

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effects of a purified substance on fungal growth, MIC values and conidial germination of *Colletotrichum gloeosporioides* as well as encysted zoospore germination of *Phytophthora parasitica*.

MATERIALS AND METHODS

Production, extraction and purification of antibiotics

S. fradiae strain SU-1 (Apichaisataienchote, 2005) was cultured in a 10 litre scale of YMG liquid medium on a rotary shaker with 95 rpm at 28 °C for four days. The antifungal activity against *C. gloeosporioides* was extractable with ethyl acetate (EtOAc) and found to be present both in the culture broth and in the mycelia. A sequence of chromatographic steps starting with Sephadex LH-20 (Pharmacia) and followed by silica gel column chromatography yielded 0.44 mg of aerugine. A fraction of the crude extract was used for primary bioassays against plant pathogenic fungi, namely *Colletotrichum gloeosporioides, Fusarium moniliforme, Phytophthora parasitica,* and *Pythium* sp. by the paper disk-agar diffusion method.

Instrumental analysis

^{1}H and ^{13}C NMR spectra were measured on a Varian Unity 300 Spectrometer (300.145 MHz). ESI mass spectra were recorded on a Quattro Triple Quadrupol Mass Spectrometer, Finnigan TSQ 7000 with nano-ESI API ion source. EIMS was performed on a Finnigan MAT95 (70 eV) and perfluorokerosene was used as reference substance in EI HRMS. Flash chromatography was carried out on silica gel 60: 0.05-0.2 mm, 70-270 mesh (Macherey-Nagel & Co., Düren). Thin layer chromatography (TLC) was performed on Polygram SIL G/UV$_{254}$ (Macherey-Nagel & Co.). R_f values were measured on Polygram SIL G/UV$_{254}$ (Macherey-Nagel & Co.). Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia).

Biological assay

Minimum inhibitory concentrations (MIC) of aerugine were determined in sterile, flat-bottomed 96-well microtitre plates (Nucleon™, Denmark). Spore and zoospore suspensions (~ 10^5 spores/ml) of *C. gloeosporioides* and *P. parasitica* were used. 10 µl of the spore suspensions were added to each well containing 1 ml of malt extract broth supplemented with 1 µl of aerugine at the concentrations of 12.5, 25, 50, 75 and 100 µg/ml. The lowest concentrations of aerugine that completely inhibited hyphae formation were considered as MICs (Handelsman *et al*., 1991). Conidia and zoospore germination were also recorded 12 and 24 h after inoculation using stereomicroscopy and light microscopy. Commercial antifungal and antioomycetal compounds such as chlorothalonil and metalaxyl were used as references at the concentrations of 12.5 to 150 µg/ml (Kim *et al*., 2000).

RESULTS AND DISCUSSION

Ethyl acetate extract showed antifungal activity against *C. gloeosporioides, F. moniliforme, P. parasitica,* and *Pythium* sp. by using the agar diffusion method. The extract inhibited radial growth of *C. gloeosporioides* and *P. parasitica*. Active fractions from Sephadex LH-20 and silica gel afforded a yellow oil that showed strong UV absorption at 254 nm and a blue fluorescence under UV light at 366 nm on TLC. It exhibited no colour reaction with anisaldehyde/sulphuric acid reagent (1 ml anisaldehyde was added to a solution of 85 ml methanol, 14 ml acetic acid and 1 ml sulphuric acid.). However, it gave a weakly brown spot with aqueous $PdCl_2$, indicating a sulphur content. The proton NMR spectrum (Figure 1) displayed four aromatic protons as two *pseudo*triplets at δ 7.34 (4-H) and 6.90 (5-H), one doublet of doublet at δ 7.41 (6-H) and a broad doublet at δ 7.00 (3-H), indicating an *ortho*-disubstituted benzene ring. The aliphatic region exhibited a methine proton as a multiplet at δ 4.88 (4′-H), and two methylene

groups in the range of δ 3.30-4.00. The carbon NMR spectrum indicated the presence of 10 signals, assigned as four aromatic methines, two methylene signals, one methine group, and three quaternary carbon signals.

The EI and ESI mass spectra (Figure 2) delivered the molecular weight *m/z* 209. A search in AntiBase (Laatsch, 2003) with all these information (Figure 1 and 2) led to the identification of the purified constituent as aerugine (Figure 3).

The sulphur-containing aerugine (4-hydroxymethyl-2-(2-hydroxyphenyl)-2-thiazoline) was previously reported to be obtained

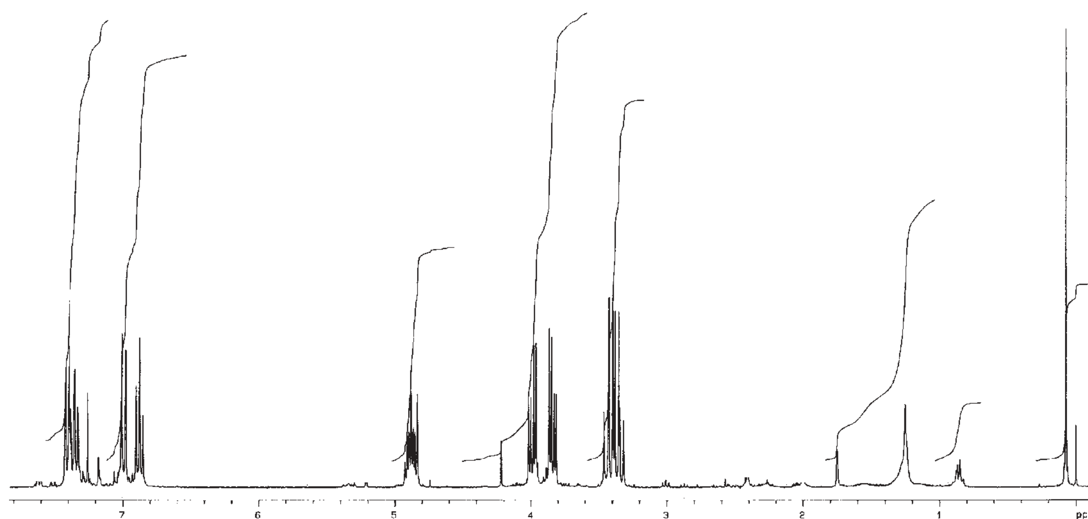

Figure 1 ^{1}H NMR spectrum (300 MHz) of aerugine in $CDCl_3$. Chemical shifts as δ values from 0 (right) to 8 ppm (left).

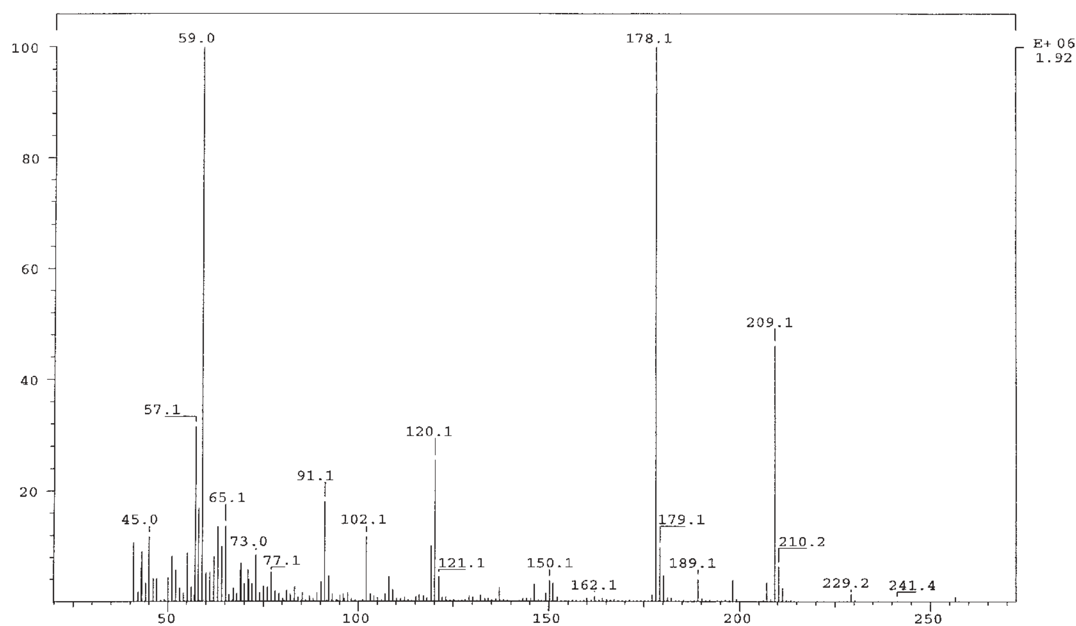

Figure 2 EI Mass spectrum (70 eV) of aerugine.

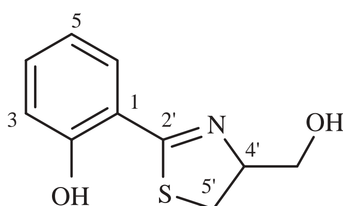

Figure 3 Chemical structure of aerugine.

from *Pseudomonas aeruginosa* strain 590 (Zunnundzhanov *et al*., 1987). Lee *et al.* (2003) isolated aerugine from *Pseudomonas fluorescens* strain MM-B16 and found activities against *Colletotrichum orbiculare* and *Phytophthora capsici.* Thiazoline compounds have demonstrated a wide range of pharmacological activities and flavour properties (Fernandez *et al.*, 2000). Further thiazoline derivatives were isolated from cyanobacteria, bacteria and other streptomycetes, eg. curacin D from *Lyngbya majuscula* (Marquez *et al.*, 1998), thiangazole from *Polyangium* sp. (Kunze *et al.*, 1993), desferrithiocin from *Streptomyces antibioticus* (Mulqueen *et al.*, 1993), bleomycin from *Streptomyces verticillus* ATCC15003 (Liangcheng *et al.*, 2000)**,** watasemycins A and B from *Streptomyces* sp. TPA0597 (Sasaki *et al.*, 2002). However, production of aerugine from the genus *Streptomyces* has not been reported. In addition, there is little information on the isolation and biological properties of aerugine and its analogues from other microorganisms. This is the first report of aerugine isolated from the culture of *Streptomyces fradiae* strain SU-1.

Bioassay

The minimum inhibitory concentration of aerugine against *C. gloeosporioides* and *P. parasitica* was 12.5 μg/ml. A bioassay with the commercial antifungal and antioomycete agents chlorothalonil and metalaxyl as standards was carried out for comparison. Aerugine completely inhibited conidia germination of *C. gloeosporioides*, causing chili anthracnose, after 12 h of incubation and inhibited of encysted 100% zoospore germination of *P. parasitica,* causing buckeye rot of tomato and root rot of tangerine, after 24 h of incubation (Table 1).

Due to the strong antifungal and antioomycete activity, aerugine isolated from the culture of *S. fradiae* SU-1 would be feasible for agricultural use. The *in vivo* control of *C. gleosporioides* on chilli and *P. parasitica* on tomato and tangerine by aerugine should, therefore, be further tested in the greenhouse and in the field. In addition, a formulation of *S. fradiae* as a biological control agent would be developed. The feasibility of using a microbial biological control agent (BCA) in the greenhouse or the field was determined largely by its formulation, shelf life, and delivery technologies. However, the research on the formulation of actinomycetous

Table 1 Inhibition effect of aerugine on conidia germination (%) of *Colletotrichum gloeosporioides* and encysted zoospore germination of *Phytophthora parasitica.*

Concentration of aerugine (ppm)	Inhibition of conidial * germination (%)		Inhibition of encysted zoospore germination (%)*	
	12h	24h	12h	24h
0	35.0	0	55.0	0
12.5	100.0	100.0	81.8	100.0
25.0	100.0	100.0	92.7	100.0
50.0	100.0	100.0	94.5	100.0
100.0	100.0	100.0	94.5	100.0

* inhibition of conidia and encysted zoospore germination (%) = mean average from 2 fields of microscopic view at magnification of 400x

bacteria as commercial biofungicides or bioinsecticides is limited. So far only *Streptomyces griseoviridis*, Mycostop (Kemira Agro Oy, Helsinki, Finland), has been commercially available in Europe and North America as a wettable powder for protection of greenhouse ornamental and vegetable crops against soil borne fungal pathogens, in particular *Alternaria*, *Botrytis*, *Fusarium*, and *Phomopsis* (Tahvonen and Avikainen, 1987) and possibly for *Pythium*, *Phytophthora* and *Rhizoctonia* (Tahvonen and Lahdenperae, 1988).

CONCLUSION

An antifungal and antioomycete antibiotic, aerugine, was isolated from the culture filtrate of *S. fradiae* strain SU-1 and extracted by ethyl acetate. The extract strongly inhibited radial growth of *Colletotrichum gloeosporioides* and *Phytophthora parasitica* in the agar diffusion test. Aerugine was purified by Sephadex LH-20 and silica gel column chromatography. The structure was determined by detailed interpretation of ^{1}H, ^{13}C NMR and mass spectra. The minimum inhibitory concentrations of aerugine were 12.5 µl/ml determined against both *C. gloeosporioides* and *P. parasitica*. It completely inhibited conidial germination of *C. gloeosporioides* after 12 h of incubation and 100% inhibited encysted zoospore germination of *P. parasitica* after 24 h of incubation.

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LITERATURE CITED

Apichaisataienchote, B., J. Altenbuchner and H. Buchenauer.2005. Isolation, identification of *Streptomyces fradiae* SU-1 from Thailand and protoplast transformation with the chitinase B Gene from *Nocardiopsis prasina* OPC-131. **Current Microbiol.** 51: 116-121.

Baltz, R.H., E.T. Seno, J. Stonesifer P. Matsushima and G.M. Wild.1981.Genetics and biochemistry of tylosin production by *Streptomyces fradiae*. pp.371- 375. *In* D. Schelesinger (ed.). **Microbiology.** American Society for Microbiology. Washington D.C.

Bossi, R., R. Hutter, W. Keller-Schierlein, L. Neipp, and H. Zahner. 1958. Metabolic products from Actinomycetes. XIV. Actinomycin Z. Helv. **Chim. Acta.** 41:1645-52.

Drautz, H., H. Zaehner, J. Rohr and A. Zeeck. 1986. Metabolic products of microorganisms. Urdamycins, new angucycline antibiotics from *Streptomyces frdiae*. Isolation, characterization and biological properties. **J. Antibiot.** 39: 1657-1669.s

Fernandez, X., R. Fellous and E. Dunach. 2000. Novel synthesis of 2-thiazoline.**Tetrahedron Lett.** 41: 3381-3384.

Handelsman, J., W.C. Nesmith and S.J. Raffel. 1991. Microassay for biological and chemical control of infection to tobacco by *Phytophthora parasitica* var. *nicotianae*. **Current Microbiol.** 22 (5): 317-319.

Hewitt, H.G. 1998. Natural products and the biological control of crop disease pp. 190 *In* **Fungicides in Crop Protection**. CAB International, UK.

Hickey, R. J. and P.H. Hidy. 1951. Crystalline fradicin. **Science** 113: 361-362.

Igarashi, M., K. Ogata and A. Miyake. 1955. *Streptomyces*: Fradicin-mycelin group substance. **J. Antibiot.** 113-117.

Kim, B.S., J.Y. Lee and B.K. Hwang. 2000. *In vivo* control and *in vitro* antifungal activity of rhamnolipid B, a glycolipid antibiotic, against *Phytophthora capsici* and *Colletotrichum orbiculare*. **Pest Manag. Sci.** 56: 1029-1035.

Kunze, B., R. Jansen, L. Pridzum, E. Jurkiewicz, G. Hunsmann, G. Hofle and G. Reichenbach. 1993. Thiazoline antibiotic from *Polyangium sp.* (myxobacteria): production, antimicrobial activity and mechanism of action. **J. Antibiot.** 46:1752-1755.

Laatsch, H. Antibase.2002.A Natural products database for rapid structure determination. Chemical Concepts, Weinheim 2002; Internet http://www.gwdg.de/~ucoc/Laatsch/

Lackner, H., I. Bahner, N. Shigematsu, L.K. Pannell and A.B. Mauger. 2000. Structure of five components of the actinomycin z complex from *Streptomyces fradiae*, two of which contain 4-chlorothreonine. **J. Nat. Prod.** 63: 352-356.

Lee, J.Y., S.S. Moon and B.K. Hwang. 2003. Isolation and antifungal and antioomycete activities of aerugine produced by *Pseudomonas fluorescens* strain MM-B16. **Appl. Environ. Microbiol.** 69: 2023-2031.

Liangcheng, D., C. Mei, S. César and S. Ben. 2000. An oxidation domain in the BlmIII non ribosomal peptide synthetase probably catalyzing thiazole formation in the biosynthesis of the anti-tumor drug bleomycin in *Streptomyces verticillus* ATCC15003. **FEMS Microbiol. Lett.** 189:171-175.

Marquez, B., P. Verdier-Pinard, E. Hamel and W.H. Gerwick. 1998. Curacin D, an antimitotic agent from the marine cyanobacterium *Lyngbya majuscula*. **Phytochem.** 49:2387-238.

Mulqueen, G.C., G. Pattenden and D.A. Whitting. 1993. Synthesis of the thiazoline-based siderophore (*S*)-desferrithiocin.**Tetrahedron** 49: 5359-5364.

Sasaki, T., Y. Igarashi, N. Saito and T. Furumai. 2002. Watasemycins A and B, new antibiotics produced by *Streptomyces* sp. TP-A0579. **J. Antibiot.** 55:249-255.

Stapley, E. O., D. Hendlin, M.J. Martinez, M. Jackson, H. Wallick, S. Hernandez, S. Mochales, S. Currie and R.M. Miller. 1969. Phosphonomycin. I. Discovery and *in vitro* biological characterization. **Antimicr. Agents Chemother.** 284-290.

Steven, C. B. 2002. Biological and cultural tests for control of plant diseases. available source htpp://www.apsnet.org/online/Bctests/Vol17/materailstop.htm Retrieved 23 July, 2003.

Tahvonen, R. and H. Avikainen. 1987. The biological control of seedborne *Alternaria brassicicola* of cruciferous plants with a powdery preparation of *Streptomyces sp*. **J. Agri. Sci.** Finl. 59: 199-208.

Tahvonen, R. and L. Lahdenperae. 1988. Biological control of *Botrytis cinerea* and *Rhizoctonia solani* in lettuce by *Streptomyces* sp. **An. Agric. Fenniae.** 27: 107-116.

Truong, C. Q., T.L. Chu, V.K. Nguen, T.C. Hoang, L. Mai, K.L. Ngo and H.D. Doan. 1977. A new antibiotic, dekamycin. **Biologia (Bratislava, Slovakia).** 32: 217-22.

Waksman, S. A. and H.A. Lechevalier. Neomycin. 1957. Rutgers Research and Educational Foundation. US Patent 2799620 19570716.

Waksman, S.A. and H.A. Lechevalier. 1962. Description of antibiotics. pp.206-307. *In* **The Actinomycetes vol III: Antibiotics of Actinomycetes.** The Williams & Wilkins Company, Baltimore**.**

Zunnundzhanov, A., I.A. Bessonova, N.D. Abdullaev and D.K. Ogai. 1988. Structure of aerugine from *Pseudomonas aeruginosa*. **Chem. Nat. Comp.** 23: 461-465.