Thiobutacin, a Novel Antifungal and Antioomycete Antibiotic from *Lechevalieria aerocolonigenes*

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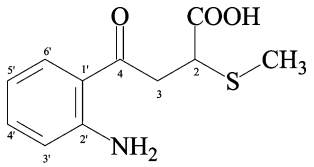
A novel butanoic acid, thiobutacin (**1**), 4-(2-aminophenyl)-4-oxo-2-methylthiobutanoic acid ($C_{11}H_{13}NO_3S$), was isolated from the culture broth of a soil actinomycete, *Lechevalieria aerocolonigenes* strain VK-A9. The structure of thiobutacin (**1**) was elucidated on the basis of the extensive 2D NMR spectral data including $^1H-^1H$ COSY, HMBC, HMQC, ROESY, and NOESY. Thiobutacin (**1**) showed antioomycete and antifungal activity against phytopathogenic *Phytophthora capsici* and *Botrytis cinerea* and the yeast *Saccharomyces cerevisiae*.

Several antibiotics, such as antibacterial antibiotics 3-*O*-isobutyrylkinamycin C and 4-deacetyl-4-*O*-isobutyrylkinamycin C,[1] antifungal antibiotic formamicin,[2] antitumor antibiotics tetrazomine[3] and rebeccamycin,[4] and herbicidal antibiotic phosphonothrixin,[5] have been isolated from the genera *Lechevalieria* (formerly classified as *Saccharothrix*) and *Saccharothrix*. However, the reports of antifungal and antioomycete antibiotics produced by the genus *Lechevalieria* are very limited. In our search for soil actinomycetes producing antifungal and antioomycete antibiotics active against the plant fungal pathogens, we have isolated thiobutacin (**1**) from the culture broth of *Lechevalieria aerocolonigenes* VK-A9. In this report, we describe the isolation and structure elucidation of this novel antibiotic, which was found to be active against phytopathogenic *Phytophthora capsici* and *Botrytis cinerea* and the yeast *Saccharomyces cerevisiae*.



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Thiobutacin (**1**) was isolated from the fermentation broth of *L. aerocolonigenes* using various chromatographic procedures. A total of 9.8 mg of pure **1**, active against *P. capsici*, was obtained as a brown solid from a single peak by preparative HPLC. Thiobutacin (**1**) was soluble in organic solvents such as MeOH, EtOH, $CHCl_3$, EtOAc, and DMSO, but poorly soluble in H_2O. The structure of the antibiotic **1** was determined by analyses of mass, IR, and NMR spectral data. The EI mass spectrum of **1** confirmed its molecular weight to be 239. The major peak at *m/z* 120 in the mass spectrum corresponded to a fragmentation of the aryl ketone β to the ring. The molecular formula of **1** was deduced as $C_{11}H_{13}NO_3S$ on the basis of HREIMS, which showed a molecular ion peak at *m/z* 239.0621 (M^+). The IR spectrum showed the following major absorption bands: 1018, 1215, 1583, 1616, 2846, and 2917 cm^{-1}. UV absorption maxima of **1** appeared at 227, 260, and 360 nm in MeOH. The structure of thiobutacin (**1**) was fully elucidated by ^1H NMR and ^{13}C NMR spectroscopy, DEPT experiment, and 2D NMR spectral studies ($^1H-^1H$ COSY, HMBC, HMQC, ROESY, and NOESY).

The ^1H NMR spectrum of thiobutacin (**1**) indicated the presence of four aryl protons at δ 6.60, 6.73, 7.23, and 7.78. The ^{13}C NMR spectrum had signals of aromatic carbons at δ 115.0, 117.0, 130.8, and 134.3. The ^{13}C NMR spectrum of **1** also indicated the presence of an amine group at δ 151.4. The carbonyl signal was detected at δ 199.2 in the ^{13}C NMR spectrum. These results suggested that **1** contained an aromatic group with amine and carbonyl substituents. The ^1H NMR spectrum indicated the presence of a methylene group (δ 3.27 and 3.66), and this methylene group was confirmed by the signal at δ 41.0 in the ^{13}C NMR spectrum. The carbon resonance at δ 42.9 indicated the presence of a carbon atom attached to a carboxylic acid group that resonated at δ 174.9 in the ^{13}C NMR spectrum. The presence of a methyl group bearing a sulfur atom was confirmed at δ 2.23 in the ^1H NMR and δ 13.2 in the ^{13}C NMR spectra, respectively.

The HMBC spectrum also showed the correlations of the carbonyl carbon (δ 199.2, C-4) with the methylene protons (δ 3.27, 3.66, H-3). Correlations of the carboxylic acid carbon (δ 174.9) with methylene protons (δ 3.27, 3.66, H-3) were confirmed by HMBC analysis. The HMBC analysis revealed correlations of the sulfur-bearing methyl carbon (δ 13.2) with a proton (δ 3.71, H-2). Thus, the structure of thiobutacin (**1**) was determined to be 4-(2-aminophenyl)-4-oxo-2-methylthiobutanoic acid.

Various butanoic acid derivatives that were naturally isolated or chemically synthesized have been studied for their biological activities. The derivative 3-(3-indolyl)-butanoic acid has shown not only activity against the bacterial wilt pathogen *Ralstonia solanacearum* but also algicidal activity.[6] The antimicrobial activity of L-2-amino-4-(2-aminoethoxy)butanoic acid,[7] glutamine synthetase-inhibiting activity of 2-amino-4-(methylphosphinyl)butanoic acid and its 2-oxo-derivative,[8] antitumor activity of 2-amino-2-methylbutanoic acid,[9] and growth-inhibiting activity of 2-amino-3-cyclopropylbutanoic acid in lettuce

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seedling[10] have also been demonstrated, but there is still little information about antifungal and antioomycete activity of butanoic acid derivatives. To our knowledge, this is the first report of isolation of the antibiotic thiobutacin (**1**) from *L. aerocolonigenes* active against an oomycete plant pathogen. Thiobutacin (**1**) showed antioomycete activity against *Phytophthora capsici* in microtiter broth dilution assay (MIC 10 μg/mL). This compares well with the MIC value of 30 μg/mL obtained for the antioomycete standard metalaxyl. Thiobutacin (**1**) also exhibited antifungal activity against *Botrytis cinerea* (MIC 50 μg/mL) and the yeast *Saccharomyces cerevisiae* (MIC 30 μg/mL). Reference compounds nystatin and miconazole showed activity against *B. cinerea* at MICs of 3 and 10 μg/mL and against *S. cerevisiae* with MICs of 7 and 5 μg/mL.

Experimental Section

General Experimental Procedures. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX 500 MHz NMR spectrometer (Billerica, MA). ^{1}H NMR spectra were measured in deuterated methanol (CD_3OD) at room temperature. Chemical shifts are given in δ values (ppm) referenced to the methyl group of CD_3OD at 3.30 ppm as an internal standard. ^{13}C NMR spectra were recorded in CD_3OD. The DEPT spectrum was measured in CD_3OD with a pulse width of $\theta = 135°$. Chemical shifts of the ^{13}C NMR and DEPT spectra are given in δ values (ppm) referenced to the methyl group of CD_3OD at 49.9 ppm in CD_3OD. The ^{1}H$-^1$H COSY spectrum was recorded in a phase-sensitive, double-quantum mode using the standard pulse sequence in CD_3OD. HMBC, HMQC, ROESY, and NOESY spectroscopies were performed on a Bruker AMX 500 NMR spectrometer. Low-resolution mass spectra were recorded on a VG70-VSEQ mass spectrometer (VG Analytical, Manchester, UK) using the electron impact (EI) electron ionization method. High-resolution mass spectra were recorded on a JEOL JMS-HX110/110A tandem mass spectrometer (JEOL, Peabody, MA). FT-infrared spectroscopy was done on a Biorad instrument (Randolph, MA) using a KBr pellet. The UV spectrum was measured with a Beckman DU650 spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

Isolation and Taxonomy of Antibiotic-Producing *Lechevalieria aerocolonigenes* Strain VK-A9. The actinomycete *L. aerocolonigenes* strain VK-A9, which showed strong in vitro antifungal and antioomycete activity against plant pathogens such as *Alternaria mali*, *Colletotrichum gloeosporioides*, *Magnaporthe grisea*, *P. capsici*, and *Rhizoctonia solani*, was isolated from radish field soil in Ko-yang, Korea.[11] *L. aerocolonigenes* strain VK-A9 was cultured in a yeast-malt extract agar (YMA) (10 g of malt extract, 4 g of yeast extract, 4 g of glucose, 20 g of agar, 1 L of H_2O, pH 7.3) at 28 °C. For long-term maintenance, *L. aerocolonigenes* strain VK-A9 was preserved in a yeast-malt extract broth containing 15% glycerol at −70 °C.

For taxonomic identification, chromosomal DNA was isolated from the liquid culture of strain VK-A9 by a lysozyme, SDS, and proteinase K cell lysis, followed by chloroform and 2-propanol precipitation. The 16S rRNA genes were amplified using PCR with universal primers.[12] 16S rDNA nucleotide sequences of the strain VK-A9 were in good accordance with those of *L. aerocolonigenes*, indicating that the 16S rDNA sequence similarity of the two microorganisms was almost 99.9%. Thus, on the basis of 16S rDNA sequence analysis, we assigned the actinomycete strain VK-A9 to *L. aerocolonigenes*. The strain VK-A9 was deposited in 2003 with the Korean Collection for Type Cultures (KCTC) in Seoul, Korea, and assigned the KCTC deposition number KCTC 10461.

Extraction and Isolation of Thiobutacin (1). The 5 mL inoculum of *L. aerocolonigenes* strain VK-A9 precultured in yeast-malt extract broth at 28 °C for 2 days was transferred into 500 mL of glycerol peptone broth (20 g of glycerol, 10 g of polypeptone, 5 g of meat extract, 1 L of H_2O, pH 7.0) for the production of antibiotics. The fermentation was carried out at 28 °C for 8 days on a rotary shaker with an agitation rate of 150 rpm. Finally, a total of 78 L of cultures of *L. aerocolonigenes* strain VK-A9 were obtained for further isolation of antibiotic compounds. The culture filtrates of *L. aerocolonigenes* strain VK-A9 were combined and absorbed on a Diaion HP-20 column (6 L). The HP-20 column was eluted with stepwise gradients of MeOH in H_2O (0%, 20%, 40%, 60%, 80%, and 100%). The 60% and 80% MeOH fractions showing antifungal activity were pooled and concentrated to 4 L, followed by adjusting to pH 5. Solvent extraction was performed using an equal volume of EtOAc three times, and the concentrated EtOAc extracts were subjected to C_{18} reversed-phase flash chromatography (Lichroprep RP-18, 40−63 μm, Merck). After washing three times with 3 L of H_2O, the RP-18 column was eluted with stepwise gradients of MeOH in H_2O (0%, 20%, 40%, 60%, 80%, and 100%). The 60% and 80% MeOH fractions were absorbed on a silica gel flash column (silica gel 60, 23−660 μm mesh, Merck), and the column was eluted with stepwise gradients of $CHCl_3$ in MeOH (100%, 90%, 80%, 70%, 50%, 30%, and 10%). The 100% and 90% $CHCl_3$ fractions were loaded on a Sephadex LH-20 column (26 × 950 mm, C26/100, Pharmacia), which was eluted with MeOH. The active fractions were combined and subsequently subjected to HPLC on a C_{18} reversed-phase column. The preparative HPLC was performed with a linear gradient from 10% to 50% CH_3CN in H_2O containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 2 mL/min over 30 min.

Bioassay Procedures. Antimicrobial activity of thiobutacin (**1**) was assessed against fungi, oomycetes, and yeasts in a 24-well microtiter dish (Cell Wells, Corning Glass Works, Corning, NY) using a modified method of Nair et al.[13] Spore suspensions (10^5 spores/mL) of *B. cinerea*, *Colletotrichum orbiculare*, and *P. capsici* and cell suspensions (10^4 CFU/mL) of *Candida albicans* and *Saccharomyces cerevisiae* were prepared as inocula. A 10 μL suspension of each inoculum was added to each well containing 1 mL of potato dextrose broth (Difco). Thiobutacin (**1**) in MeOH, ranging in concentration from 0 to 100 μg/mL, was dispensed into microtiter plates. The plates were incubated at 28 °C for 2−5 days and compared with a positive control containing culture broth and microorganisms without the antibiotic. The lowest concentrations of thiobutacin (**1**) where no growth of microorganisms was observed were considered to be minimum inhibitory concentrations (MICs). Nystatin (Sigma), miconazole (Sigma), and metalaxyl (Sungbo Chemical Co, Korea) were used as reference compounds.

Thiobutacin (1): brown solid; IR ν_{max} (KBr) 1018, 1215, 1583, 1616, 2846, and 2917 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) δ 7.78 (1H, dd, J = 8.0; 1.0, H-6′), 7.23 (1H, ddd, J = 8.5; 7.0; 1.5, H-4′), 6.73 (1H, dd, J = 8.5; 1.0, H-3′), 6.60 (1H, ddd, J = 8.0; 7.0; 1.0, H-5′), 3.71 (1H, dd, J = 10.5; 3.0, H-2), 3.66 (1H, dd, J = 16.5; 10.5, H-3), 3.27 (1H, dd, J = 16.5; 3.0, H-3), 2.23 (3H, s, S-CH_3); ^{13}C NMR (CD_3OD, 125 MHz) δ 199.2 (C-4), 174.9 (COOH), 151.4 (C-2′), 134.3 (C-4′), 130.8 (C-6′), 117.0 (C-1′ and C-3′), 115.0 (C-5′), 42.9 (C-2), 41.0 (C-3), 13.2 (S-CH_3); HREIMS *m/z* 239.0621 [M$^+$] (calcd for $C_{11}H_{13}NO_3S$, 239.0616).

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