J. Antibiot. 58(1): 74–78, 2005

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THE JOURNAL OF ANTIBIOTICS

A New Antibiotic, Antimycin A_9, Produced by *Streptomyces* sp. K01-0031

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Received: August 30, 2004 / Accepted: December 2, 2004

Abstract A new antimycin group antibiotic, antimycin A_9, was isolated from a cultured broth of *Streptomyces* sp. K01-0031 together with antimycins A_{3a}, A_{3b}, A_4, and A_7, and flazin methyl ester. Antimycin A_9 is the first antimycin having an aromatic 8-acyl residue. It showed potent nematocidal and insecticidal activities against *Caenorhabditis elegans* and *Artemia salina*, respectively. It inhibited bovine heart NADH oxidase at nanomolar level like other known antimycins.

Keywords antimycin, nematocidal, insecticidal, antibiotic

Microorganisms produce many useful anthelmintic and insecticidal antibiotics [1, 2]. In the course of screening for these antibiotics, we have isolated some new compounds from microbial metabolites [3〜6]. Further screening for antibiotics using *Caenorhabditis elegans* and *Artemia salina* led to the isolation of a new antibiotic, antimycin A_9 (**1**, Fig. 1), which was produced by a cultured broth of *Streptomyces* sp. K01-0031. In this report, we describe taxonomy of the producing strains and fermentation, isolation, structure elucidation, and biological properties of antimycin A_9.

The strain K01-0031 was isolated from a mountain soil sample collected at Chuxiong, Yunnan, China. The International *Streptomyces* Project (ISP) media recommended by Shirling and Gottlieb [7] and media recommended by Waksman [8] were used to investigate the cultural and physiological characteristics. Cultures were routinely observed after the incubation for two weeks at 27°C. The utilization of carbon sources was tested by growth on Pridham and Gottlieb's medium containing 1% carbon at 27°C [9]. The vegetative mycelia grew abundantly on yeast extract - malt extract agar, glycerol-calcium malate agar, and other agar media, and did not show fragmentation into coccoid forms or bacillary elements. The aerial mycelia grew abundantly on yeast extract - malt extract agar. The morphological properties were observed with a JEOL JSM-5600 scanning electron microscope. The spore chains were straight and each had more than 20 spores per chain. The spores were cylindrical in shape, 1.1〜1.3×0.5〜0.6 μm in size, and had a smooth surface (Fig. 2). Whirls, sclerotic granules, sporangia, and flagellate spores were not observed. The type of

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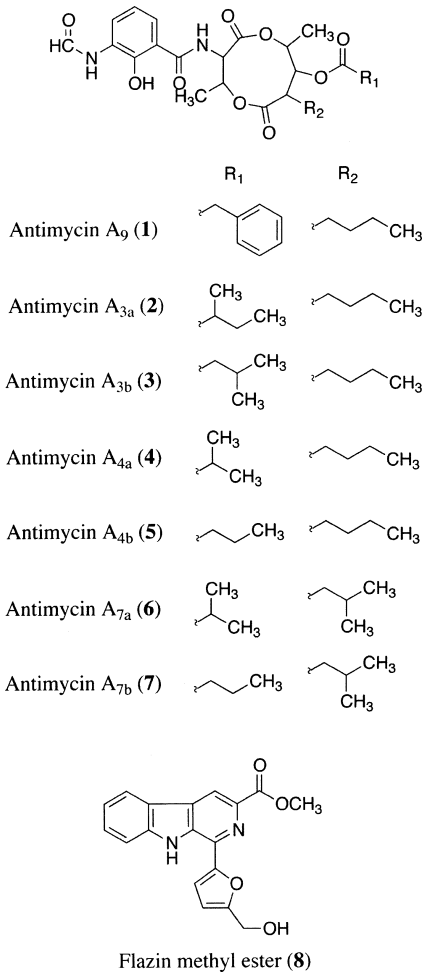

Fig. 1 Structures of antimycins (**1**～**7**) and flazin methyl ester (**8**).

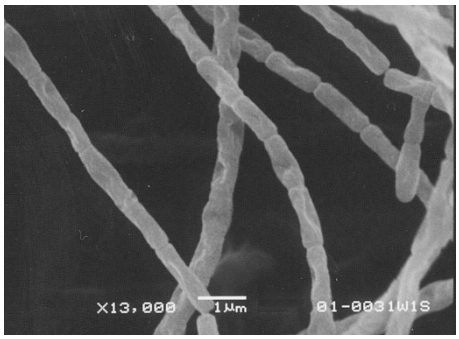

Fig. 2 Scanning electron micrograph of spore chains of strain K01-0031 grown on sucrose-nitrate agar at 27°C for 14 days.

diaminopimelic acid (DAP) isomers was determined by the method of Becker *et al.* [10]. The isomer of DAP in whole-cell hydrolysates of strain K01-0031 was determined to be the LL-form. Menaquinones were extracted and purified after Collins *et al.* [11] and then analyzed by HPLC equipped with a Capcell Pak C18 column (Shiseido Co.) [12]. Major menaquinones were MK-9(H_6) and MK-9(H_8). The color of vegetative mycelia showed brown and the aerial mass color showed white to gray. Melanoid and brownish pigments were produced. D-Glucose, L-arabinose, D-xylose, raffinose, melibiose, D-mannitol, D-fructose, *myo*-inositol, and sucrose were utilized for growth, but L-rhamnose was not. The temperature range for growth was 3～37°C. Based on the taxonomic properties described above, strain K01-0031 is considered to belong to the genus *Streptomyces* [13].

A stock culture of *Streptomyces* sp. K01-0031 was inoculated into a large test tube containing 10 ml of a seed medium consisted of glucose 0.1%, starch (Hokuren Federation of Agricultural Cooperatives) 1.0%, peptone (Kyokuto Pharmaceutical Industrial Co.) 0.3%, meat extract (Kyokuto Pharmaceutical Industrial Co.) 0.3%, yeast extract (Oriental Yeast Co.) 0.1%, and $CaCO_3$ 0.3%, pH 7.0. The fermentation was carried out on a reciprocal shaker at 27°C for 2 days. One milliliter of the first seed culture was transferred into each of eight 500-ml Erlenmeyer flasks containing 100 ml of the same seed medium and incubated on a rotary shaker at 27°C for 3 days. Then 400 ml of the second seed culture was transferred into each of two 30-liter jar fermenters containing 20 liters of a production medium consisted of glucose 0.5%, corn steep powder (Marcor Development Co.) 1.0%, oatmeal (Nihon Shokuhin Seizo Co.) 1.0%, Pharmamedia (Traders Protein) 1.0%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, allophane (Shinagawa Chemicals Co.) 0.5%, $FeSO_4 \cdot 7H_2O$ 0.0001%, $MnCl_2 \cdot 4H_2O$ 0.0001%, $ZnSO_4 \cdot 7H_2O$ 0.0001%, $CuSO_4 \cdot H_2O$ 0.0001%, and $CoCl_2 \cdot 6H_2O$ 0.0001%, pH 7.0. It was cultured at 27°C for 4 days.

The whole cultured broth (40 liters) was extracted with acetone, which was then removed from the extract by evaporation. The aqueous extract was partitioned with $CHCl_3$, and the organic layer was concentrated to dryness *in vacuo* to afford crude active material (14.4 g), that was chromatographed over a silica gel column. Active fractions eluting with toluene - acetone (5 : 1) were concentrated to yield a crude material (1.07 g), that was applied to an ODS silica gel column and eluted with aqueous methanol. Elution using 80% methanol afforded a mixture of antimycins (59.8 mg). They were applied onto a Pegasil ODS HPLC (i.d. 20×250 mm, Senshu Scientific Co.) and eluted with 70% aqueous-CH_3CN. A white powder of antimycin A_9 (**1**, 5.8 mg) was purified together with antimycins A_{3a} (**2**, 4.2 mg), A_{3b} (**3**, 20.2 mg) [14, 15], A_4

Table 1 ^{1}H and ^{13}C data (in $CDCl_3$) of **1**[a]

Position	δ_C (mult)	δ_H (int, *J* (Hz))
2	170.1 s	
3	53.6 d	5.27 dd (1H, 7.7, 7.7)
4	70.9 d	5.71 dq (1H, 6.6, 7.7)
6	172.8 s	
7	50.1 d	2.48 dt (1H, 10.7, 10.7)
8	76.0 d	5.04 dd (1H, 9.6, 10.0)
9	74.8 d	4.93 dq (1H, 6.3, 9.6)
4-CH_3	14.9 q	1.30 d (3H, 6.6)
9-CH_3	17.7 q	1.15 d (3H, 6.3)
1′	112.6 s	
2′	150.6 s	
3′	127.46 s	
4′	124.8 d	8.55 d (1H, 8.0)
5′	119.0 d	6.92 dd (1H, 8.0, 8.0)
6′	120.1 d	7.23 d (1H, 8.0)
1′-*C*ONH	169.4 s	
1′-CON*H*		7.05 d (1H, 7.7)
2′-OH		12.60 s (1H)
3′-NH		7.94 s (1H)
HC=O	158.9 d	8.50 s (1H)
α	27.9 t	1.60 m (2H)
β	29.2 t	1.14 m (1H), 1.60 m (1H)
γ	22.3 t	1.04 m (1H), 1.16 m (1H)
δ	13.7 q	0.81 dd (3H, 7.0, 7.0)
1″	170.0 s	
2″	41.6 t	3.66 s (2H)
3″	133.1 s	
4″, 8″	129.2 d ×2	7.289 d (2H, 7.4)
5″, 7″	128.8 d ×2	7.34 dd (2H, 7.4, 7.4)
6″	127.53 d	7.288 dd (1H, 7.4, 7.4)

[a] NMR spectra were recorded on a Varian Inova 600 spectrometer. Chemical shifts are shown in δ values (ppm) relative to $CDCl_3$ at 7.26 ppm for ^{1}H NMR and at 77.0 ppm for ^{13}C NMR.

(A_{4a} (**4**) or A_{4b} (**5**) 8.4 mg) [15, 16], and A_7 (A_{7a} (**6**) or A_{7b} (**7**), 1.4 mg) [17]. Elution using 70% methanol on the ODS silica gel column chromatography, also afforded an active material (46.5 mg), which was purified by HPLC to yield flazin methyl ester (**8**, 16.3 mg). Flazin is a constituent of Japanese soy sauce, and **8** was reported as a derivative of flazin [18].

The physico-chemical properties of **1** are as follows: HR-FAB-MS $(M+Na)^+$ *m/z* 577.2168, calcd 577.2162 for $C_{29}H_{34}N_2O_9Na$; $[\alpha]_D^{25}$ +83.6° (*c* 0.157, MeOH); mp 134~139°C; UV λ_{max}^{EtOH} nm (ε) 209 (sh, 27,300), 228 (47,700), 347 (10,100); IR ν_{max} (KBr) cm^{-1} 3415, 3350, 2929, 1745 (ester C=O), 1635 (amide C=O), 1539, 1377, 1151. Compound **1** is soluble in MeOH, acetone, acetonitrile, EtOAc, and $CHCl_3$, and insoluble in H_2O and *n*-hexane. The molecular formula of **1** was established as $C_{29}H_{34}N_2O_9$ by HR-FAB-MS. The IR spectrum of **1** is similar to those of known antimycins [16]. Chemical shifts in the ^{1}H and ^{13}C NMR of **1** are shown in Table 1. The chemical shifts of a dilactone ring, a 3-formamidosalicyl moiety, and an *n*-butyl side chain were almost the same as those of antimycins A_3 and A_4 [17]. ^{1}H-^{1}H COSY and HMBC experiments confirmed the partial structure as shown in Fig. 3. As for the 8-acyl moiety, aliphatic acyl signals were not observed, and some new signals appeared. They were assigned as a phenylacetyl residue by NMR studies (Fig. 3), and the long-range coupling between 8-H (δ 5.04) and C-1″ (δ 170.0) revealed that the phenylacetyl residue is connected to C-8 by an ester bond. Therefore, the structure of **1** was elucidated as shown in Fig. 1. Further studies are necessary to clarify its stereochemistry.

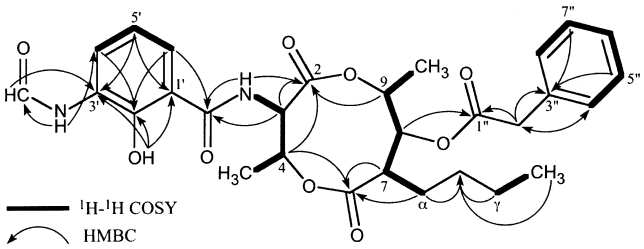

Fig. 3 Selected ^{1}H-^{1}H COSY and HMBC correlations of **1**.

Many antimycin group antibiotics have been reported until now, but all of them are compounds having aliphatic 8-acyl residues or deacylated compounds. UK-2A~UK-2D and UK-3A have benzyl side chains, but they are bonded to C-7 [19~22]. Compound **1** is the first antimycin having an aromatic 8-acyl residue.

Nematocidal and insecticidal activities of these isolated compounds were studied by a microplate assay using free-living nematode *C. elegans* and brine shrimp *A. salina*. The assay method was reported previously [7]. MIC values of **1**~**8** against *C. elegans* and *A. salina* were shown in Table 2. Antimycins were active against both organisms. Compound **1** showed slightly more potent activity than other known antimycins. Compound **8** was only weakly active against *A. salina*. Antimicrobial activity of **1** was evaluated using the following microorganisms: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* NIHJ, *Pseudomonas aeruginosa* IFO 3080, *Candida albicans* KF 1, S*accharomyces cerevisiae* KF 26, *Shizosaccharomyces pombe* IFO 0347, *Aspergillus niger* KF 103, *Mucor racemosus* IFO 4581, *Penicillium chrysogenum* KF 270, and *Trichophyton mentagrophytes* KF 331. The MIC value against *T. mentagrophytes* was

Table 2 MIC of **1**~**8** against *Caenorhabditis elegans* and *Artemia salina*.

Compound	*Caenorhabditis elegans*	*Artemia salina*
Antimycin A_9 (**1**)	0.2	0.05
Antimycin A_{3a} (**2**)	1	0.2
Antimycin A_{3b} (**3**)	1	0.2
Antimycin A_4 (**4** or **5**)	1	0.2
Antimycin A_7 (**6** or **7**)	>1	0.2
Flazin methyl ester (**8**)	>100	20

0.31 μg/ml, but the values against other microorganisms were >10 μg/ml. Antimycins are well known to inhibit ubiquinol : cytochrome c reductase (complex III) and widely used in functional studies of the enzyme [23]. Therefore, we evaluated the inhibitory activities of **1** and **3** against bovine heart NADH oxidase (Complexes I+III+IV). The assay was carried out with slight modification of the method of Mackler [24]. The IC_{50} values of **1** and **3** were 35 nM and 15 nM, respectively.

Acknowledgements We are grateful to Dr. Rokuro Masuma of Kitasato Institute for Life Sciences, Kitasato University for the evaluation of MIC of **1** against microorganisms. We also thank Ms. Akiko Nakagawa and Ms. Chikako Sakabe of School of Pharmaceutical Sciences, Kitasato University for measurements of mass spectra. K.S. was supported partly by a Grant-in-Aid for Scientific Research (14593006) from the Japan Society for the Promotion of Science. This work was also supported in part by the Grant of the 21st Century COE Program, Ministry of Education, Culture, Sports, Science, and Technology.

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