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A New Fungicide Produced by a *Streptomyces* sp. GAAS7310

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Abstract Directed bioassay guided fraction led to a new macrodiolide antimycin A_{17} (**1**), isolated from a *Streptomyces* sp. GAAS7310, which showed significant antimicrobial activity against eleven fungal species, including *Curvularia lunata* (Wakker) Boed, *Rhizopus nigrtcans* Ehrb and *Colletotrichum nigrum* EL. *et* Halst. The structure was unambiguously established by interpretation of 1D and 2D NMR data and comparison with the known antimycin A_{1a}.

Keywords *Streptomyces* sp.; antimycin; antimicrobial activity; fungicide; macrodiolide; actinomycete

Introduction

Actinomycetes are prolific producers of antibiotics and other industrially useful secondary metabolites [1~5]. Because these metabolites are often active against a limited number of specific target species, biodegradable to nontoxic products, and potentially suitable for use in integrated management programs, they can lead to the development of new classes of safer disease control agents. In our course of screening for novel naturally occurring fungicides from actinomycetes, the acetone extract of the mycelium of a *Streptomycetes* sp. 7310# collected at Tianshan mountain was found to have strong activity against 12 fungal strains, *e.g. Phomopsis vexans* (Sacc *et* Syd) Harter *etc*. By bioassay guided fractionation, we isolated a new macrodiolide, antimycin A_{17} (**1**), which showed activity against several fungi. Among these fungi, *Curvularia lunata* (Wakker) Boed, *Rhizopus nigrtcans* Ehrb and *Colletotrichum nigrum* EL. *et* Halst were more sensitive to antimycin A_{17} (**1**).

Materials and Methods

General Experimental Procedure

Melting point was determined on a XT-4 apparatus and was uncorrected. NMR spectra were recorded on a Varian Inova-500 NMR spectrometer (500 MHz) using acetone-d_6 as solvent and TMS as an internal standard. Mass spectra were measured on a VG-ZAB-HS analytical spectrometer. IR spectra were taken on a Nicolet 5DX-FTIR spectrophotometer. Optical rotation was determined in acetone at room temperature using a Horiba High Sensitivity SEPA-300 Polarimeter, and elemental analysis was recorded on an Elementar Vario EL CHNS-O elemental analysis device. Preparative HPLC were performed on an Waters 2695 system equipped with an autosampler, a quaternary pump system, a photodiode array detector, and a Chemstation data system. A 250 mm× 20 mm i.d., 5 μm particle size Zorbax Eclipse XDB ODS column (Agilent) was selected for HPLC preparation. Analytical thin-layer chromatography (TLC) was carried out on precoated silica gel aluminum plates (Merck, 60 F_{254}, 0.2 mm thickness) and HPTLC plates (Merck, RP-18). Silica gel (200~300 mesh, Qingdao Haiyang Chemical Co., Ltd.), RP-18 silica gel (50 μm, Merck), and CAD-45 absorber resin (26~60 mesh, Anhui Sanxing resin technology Co., Ltd.), were used for column

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chromatography. The solvents used for high-performance liquid chromatography (HPLC) were of HPLC grade (Sigma); and other solvents and chemicals were of analytical grade.

Collection and Cultivation

The bacterium *Streptomyces* sp. GAAS7310 was isolated from the soil collected at Tianshan mountain, Xinjiang Province, P.R. China. It has been deposited in the China Center for Type Culture Collection (CCTCC), Wuhan, P.R. China. Deposited number was CCTCC M 203058 *Streptomyces* sp. GAAS7310. Starter cultures were maintained on cornmeal agar. Plugs of agar supporting mycelial growth were cut and transferred aseptically into 250 ml Erlenmeyer flasks containing 100 ml of liquid medium (starch 1%, calcium carbonate 0.2%, ammonium sulfate 0.2%, potassium dihydrogen phosphate 0.1%, magnesium bisulfate 0.1%, sodium chloride 0.1%, yeast extract 1%, pH 7.2). The content were incubated at 28°C on a rotary shaker for 5~7 days, and the mycelium was aseptically transferred to a 500-liter fermentor containing the same culture liquid (300 liters) with the following settings: air, 1.8 liters/minute; impellor speed, 200 rpm for 3 days.

Extraction and Isolation

The 300-liter culture was filtered through Celite to separate the mycelium and culture liquid. The acetone extract of mycelium exhibited the strong activity against twelve fungal species. The 85% aqueous acetone extract of 900 g mycelium was adsorbed onto a macroporous resin (CAD-45, 50 cm×9 cm i.d.), followed by on elution with water, then ethyl acetate respectively. The ethyl acetate fraction was evaporated in vacuum at 45°C and yielded 15 g pale-yellow oil, then subjected to column chromatography over silica gel (200 g, 200~300 mesh, 80 cm×3.5 cm i.d.) and eluted with petrol (bp 60~90°C)-EtOAc mixtures of increasing 10% polarity, The 30% fraction showed strong fungicidal acitivities. This fraction (1.5 g) was further purified by means of a silica gel column (80 g, 200~300 mesh, 50 cm×2.5 cm i.d.) using chloroform/methanol (95 : 5) as the solvent system to yield a bioactive mixture. Further purified by HPLC (a 250 mm×20 mm i.d., 5 μm particle size Zorbax Eclipse XDB ODS column) with methanol/water (70 : 30) as the mobile phase to yield the active component **1** (retention time 15.1 minutes, 25 mg). The molecular structure of compound **1** was elucidated by means of mass, IR, and 1D and 2D NMR spectroscopic techniques as the new macrodiolide antimycin A_{17}.

Compound **1**: colorless crystalline solid (65 mg). mp 140~142°C. $[\alpha]_D^{20}$ +114.7 (*c* 0.002, acetone); IR (KBr) ν_{max}: 3350, 2960, 1797, 1742, 1679, 1638, 1532, 1455, 1367, 1252, 1178, 1075, 875 cm^{-1}; ^{1}H and ^{13}C NMR (see Table 1); FAB-MS *m/z*: 535 $(M+H)^+$, 521, 492, 451, 372, 265, 164, 107, 102; Anal. (%): found C 60.75, H 7.15, N 5.25, O 26.85; calcd. C 60.66, H 7.16, N 5.24, O 26.9.

Fungicidal Activity *In Vitro*

Antifungal activities of antimycin A_{17} were investigated by disk assay method, using twelve representative fungi. These fungi were grown in potato dextrose agar (PDA) media, at 28°C. The PDA plates were prepared with antimycin A_{17} (at a concentration of 100 μg/ml). Then, disks (6.0 mm in diameter) of epiphyte were placed on the plates. Similarly, disks of epiphyte were also placed on the innocuous plates (adding the same dosage sterile water) as blank comparison. All plates were incubated at 28°C for 1~3 days. Each fungus had five repeats. The results are shown in Table 2. Among these fungi, *R. nigrtcans*, *Cladosporium* sp. and *C. nigrum* were most sensitive.

Results and Discussion

The acetone extract of the mycelium was adsorbed by CAD-45, followed by washing with water and EtOAc successively. The EtOAc extract was concentrated *in vacuo*, then chromatographed on silica gel column and RP-HPLC as monitored by bioassay against fungi. This afforded the active compound antimycin A_{17} (**1**) and no other antimycins were obtained.

The molecular formula of **1** was determined to be $C_{27}H_{38}N_2O_9$ by elemental analysis and FABMS data. UV absorptions at 328 and 212 nm were assigned to a chromophore with extended conjunction. Analysis of the ^{13}C NMR spectrum (Table 1) showed five carbonyl carbon signals (δ 175.7, 174.1, 170.9, 170.3, 160.7), six olefinic carbon signals (δ 151.6, 128.6, 125.2, 122.6, 119.3, 114.5). These accounted for eight of the ten degrees of unsaturation required by the molecular formula, therefore illustrating compound **1** to be bicyclic.

The ^{1}H NMR signals at δ 8.47 (1H, dd, *J*=8, 1.5 Hz, H-6′), δ 7.77 (1H, dd, *J*=8, 1.5 Hz, H-4′) and δ 6.92 (1H, dd, *J*=8, 8 Hz, H-5′) indicated the presence of an ortho-trisubstituted benzene ring. In the H-H COSY, the proton correlations of NH-3′ and H-8′, and in the HMBC the correlations of the proton of NH-3′ and C-8′ suggested the presence of a NH–CHO moiety. A pentyl side chain was revealed by the contiguous correlations from H-12 to H-16 in ^{1}H-^{1}H COSY and correlations from H-12 to C-6 and C-7 in HMBC. A contiguous sequence from H-18 to H-20 and

Table 1 NMR data of antimycin A_{17} (actone-d_6,TMS)

No.	^{13}C	^{1}H (mult, J)	COSY	HMBC
2	170.3			
3	54.7	5.41 (dd, 8.0, 7.0 Hz)	NH-3, H-4	2, 4
4	72.2	5.70 (dq, 7.0, 7.0 Hz)	H-3, H-11	2, 6
6	174.1			
7	51.1	2.55 (m)	H-8	6, 12
8	76.3	5.0 (m)	H-7, 9	6, 17
9	74.8	4.9 (m)	H-8, H-10	2, 8, 10
10	18.2	1.30 (d, 7.0 Hz)	H-9	8, 9
11	15.4	1.40 (d, 7.0 Hz)	H-4	3, 4
12	29.7	1.29 (m), 1.63 (m)	H-13, 7	6, 7
13	23.1	0.95 (m), 1.12 (m)	H-12, 14	15
14	36.7	1.28 (m)	H-13, 15	16
15	28.6	1.21 (m)	H-14, 16	
16	13.9	0.86 (t, 7.0 Hz)		
17	175.7			
18	41.8	2.47 (m)	H-19, 21	19, 20
19	27.2	1.51 (m), 1.74 (m)	H-18, 20	17,18
20	11.9	1.16 (t, 7.0 Hz)	H-19	
21	17.0	1.17 (d, 7.0 Hz)	H-18	17, 18
1′	114.5			
2′	151.6			
3′	128.6			
4′	125.2	8.47 (dd, 8.0, 1.5 Hz)	H-5′	2′, 6′
5′	119.3	6.92 (dd, 8.0, 8.0 Hz)	H-4′,6′	1′, 3′, 4′, 6′
6′	122.6	7.77 (dd, 8.0, 1.5 Hz)	H-5′	2′, 4′, 5′, 7′
7′	170.9			
8′	160.7	8.52 (d, 1.5 Hz)	3′-NH	3′
NH-3′		9.09 (s)	H-8′	2′, 3′, 8′
NH-3		8.31 (d, 8.0 Hz)	H-3	3, 7′
OH-2′		12.83 (s)		

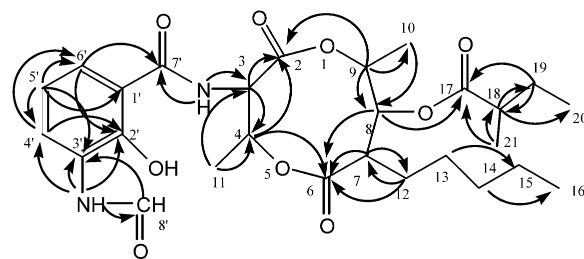

Fig. 1 Selected HMBC correlations of **1**.

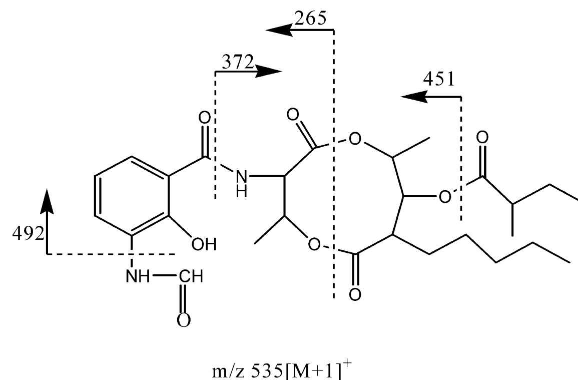

Fig. 2 Selected mass fragments of **1**.

H-21 in ^{1}H-^{1}H COSY, and by combining the HMBC correlations between H-8, H-19 and C-17, and between H-21 and C-17, C-18 respectively, established the presence of a 2-methyl butanoyl group.

The overall structure of **1** was unambiguously established by 2D NMR spectra including COSY, HMQC and HMBC (Table 1 and Fig. 1). This structure was also supported by the mass fragment patterns (Fig. 2).

In 1949, Dunshee *et al.* first isolated antimycins A from *Streptomyces* fermentation products [6]. They thought the products were consisted of at least four closely related compounds, differing only in the substituents at the 7- and 8-positions of the dilactone ring. Subsequently Abide *et al.* isolated the mixture of antimycins A into eight hitherto subcomponents, A1a, A1b, A2a, A2b, A3a, A3b, A4a and A4b by HPLC [7] and identified the structure of A1a by 1D, 2D NMR and X-ray [8, 9]. Tsunoda *et al.* had accomplished the total synthesis of antimycin A_{3b} [10]. Compared with known antimycin A series, compound **1** is different at the 7-position. In the antimycin A series, the 7-position substituent is *n*-hexyl or *n*-butyl; but in **1** is *n*-pentyl. Thus we can name **1** as antimycin A_{17} [11].

In our present study, a wide range of microorganisms was examined including 12 kinds of representative fungi. It was found that antimycin A_{17} has broad inhibitory activity to microorganisms. Especially to three fungi, *Curvularia lunata* (Wakker) Boed, *Rhizopus nigrtcans* Ehrb and *Colletotrichum nigrum* EL. *et* Halst antimycins A_{17} showed more sensitivity (Table 2). The bioassay results suggest that antimycin A_{17} may be an ideal candidate as a food preservative, pharmaceutical and natural microorganism-based product.

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Table 2 The antimicrobial activity study of antimycin A_{17} against 12 fungal strains

Fungi	Average diameter of radial growth (cm)		Rate of inhibition* (%)
	CK	Treatments	
Rhizopus nigrtcans Ehrb	7	3.5	50
Geotrichum candidum Link	6.9	6.55	5.1
Fusarium oxysporum f.sp. *cubense*	4.4	4.1	6.8
Cladosponum carpophilum Thum.	3.4	3.5	−2.9
Alternaria solani (E. *et* M.) Jones *et* Grout	5.3	3.8	28.3
Cladosporium sp.	1.5	1.4	66.7
Sclerotinia sclerotiorum (Lib.) de Bary	2	1.5	25
Peronophthora litchi *Chen*	2.8	2.5	10.7
Botrytis sp.	1.4	1.2	14.3
Colletotrichum nigrum EL.*et* Halst	2.8	1.4	50
Curvularia lunata (Wakker) Boed	3	2.2	26.7
Phomopsis vexans (Sacc *et* Syd) Harter	2.5	2.3	8

* Rate of inhibition% = [(CK average diameter of radial growth − treatments average diameter of radial growth)]/CK average diameter of radial growth × 100.

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