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A New Analog of Antimycin from *Streptomyces* sp. M03033

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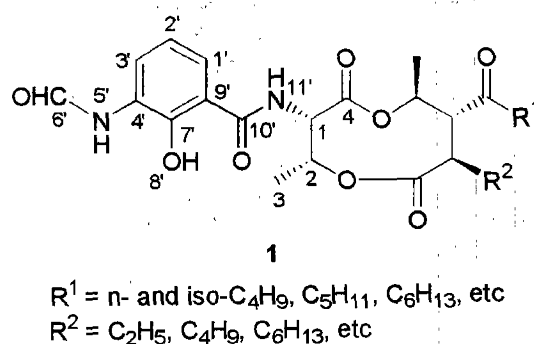
Abstract A new secondary metabolite was isolated from the culture broth and mycelium of *Streptomyces* sp. collected from marine sediment. The structure of this compound was determined to be *N*-formylantimycic acid methyl ester, an acyclic derivative of antimycin, on the basis of combined chemical and spectral methods. The structure-activity relationship of antimycins is discussed.

Key words: Antimycin, *N*-formylantimycic acid methyl ester, *Streptomyces*, structure-activity relationship

Many diseases caused by fungal infection are serious threats to humans, animals, and plants [12]. Although remarkable improvement has been achieved recently on the design and development of effective agents, the potent cytotoxicity of many compounds due to the eukaryotic nature of fungi, has been a serious limitation for a practical application of antifungal drugs [12]. The everlasting need for new antifungal agents with low side effects, i.e. toxicity, prompts for chemical investigation of living organisms for novel natural products. Organisms of marine environments have emerged as prolific sources of structurally unique and biologically active secondary metabolites [3].

As a part of the search for novel antifungal metabolites from actinomycetes collected from marine and coastal environments, we encountered a strain of *Streptomyces* from sandy sediment collected from the shore of Daebu Island, Korea, which exhibited potent antifungal activity against *Candida albicans* [2, 10]. Incubation of the strain, followed by extraction and activity-guided separation of the secondary metabolites, yielded antimycins A complex (**1**) as antifungal constituents along with a new analog [7, 8]. The structure of the novel compound was determined as *N*-formylantimycic acid methyl ester (**2**) on the basis of combined chemical and spectral analyses and comparison of the spectral data with those of metabolites belonging to the same structural class (Fig. 1) [13]. This report describes the isolation and structure elucidation of a new antimycin analog.



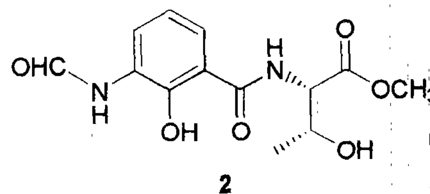


Fig. 1. Chemical structures of antimycins A complex (**1**) and *N*-formylantimycic acid methyl ester (**2**).

MATERIALS AND METHODS

General Experimental Techniques

Melting point was measured on a Fisher-Johns apparatus and is uncorrected. The HRFAB-MS spectra were obtained using a Jeol JMS-HX 110 mass spectrometer provided by the Korea Basic Science Institute, Taejon, Korea. The UV spectra were obtained in methanol using a Milton-Roy spectrophotometer. The IR spectra were recorded on a Mattson GALAXY spectrophotometer. The optical rotations were measured on a JASCO digital polarimeter using a 5-cm cell. The NMR spectra were recorded in $CDCl_3$ solutions

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on a Varian Unity 500 spectrometer. The proton and carbon NMR spectra were measured at 500 and 125 MHz, respectively. All chemical shifts were recorded according to an internal Me_4Si. The GC analysis was performed on a HP5890-II plus chromatograph equipped with an Omegawax-320 capillary column (0.32 mm×30 m, Supelco) and FID as a detector. All solvents used were of spectral grade or distilled from glass prior to use.

Microorganisms

Isolated strains of marine origin were grown in sea water media (75% sea water, 5 g bactotryptone, 3 g yeast extract, 3 ml glycerol) or SYP media at 27°C with shaking (150 rpm) for 7 days. A strain of *Candida albicans* (KCTC1940) was obtained from KCTC, Korea.

Taxonomic Studies of Strain M03033

For the identification of the strain, its taxonomical properties were examined according to "Bergy's Manual of Systematic Bacteriology" and its physiological characteristics including the utilization of carbon sources were analyzed by the method developed by Pridham and Gottlieb [6, 11].

For the fatty acid composition, the mycelium of M03033 (dry weight 0.1 g) was harvested with centrifugation, extracted with a Folch solution ($CHCl_3$:MeOH=2:1, 3×2 ml) and saponified. Fatty acid methyl esters were prepared with 5% HCl in MeOH at 80°C for 1 h, extracted with *n*-hexane, concentrated, and then analyzed using a gas chromatograph (HP5890-IIplus, Hewlett-Packard Co., U.S.A.) equipped with Omegawax-320 capillary column (0.32 mm×30 m, Supelco, U.S.A.) and FID as detector. The temperatures of injector, oven, and detector were 300, 180, and 300°C, respectively. Peaks were recorded through the Chromate data interface program (Interface Co., Korea) and analyzed with their equivalent chain length (ECL) value, and then compared with those of the authentic fatty acid methyl esters [2, 10].

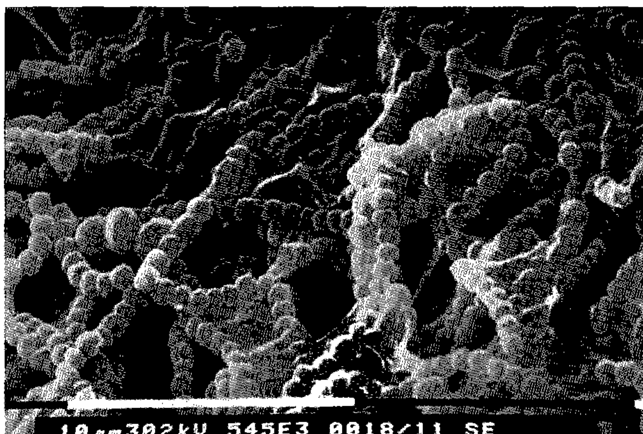

Fig. 2. Scanning electron micrograph of *Streptomyces* sp. M03033 cultured in ISP-4 medium.
White bar represents 10 μm.

Antifungal Activity

The antifungal activity was determined with the agar diffusion method using a 6-mm paper disk and the activity was measured according to the diameter of the clear zone. For activity against the mycelial phase, the test organism, *Candida albicans*, was incubated in a CO_2 incubator under 5% CO_2 atmosphere at 37°C for 2 days. After placing a paper disk with 10 mg of the compounds, the agar plate was incubated for 1 day, and the clear zone was measured.

***N*-Formylantimycic Acid Methyl Ester (2)**

HRFAB-MS *m/z* $(M+H)^+$ 297.1060 (calcd *m/z* 297.1087 for $C_{13}H_{17}O_6N_2$); mp 127–130°C; IR ν_{max} (KBr) cm^{-1} 3300, 1735, 1670, 1640, 1545, 1435, 1220; UV λ_{max} (MeOH) nm (log ε) 326 (3.62), 229 (4.36); $[\alpha]_D^{25}$ +6.0° (*c* 0.29, MeOH); ^1H and ^{13}C data, see Table 4.

Table 1. Basic taxonomical properties of *Streptomyces* sp. M03033.

Characteristics	M03033
Colony morphology on ISP 2 and 4	
Periphery	Limited, infinite
Surface	Wrinkled, flat
Aerial mycelium	Abundant, thin
Spore mass color	Ivory, greenish-ivory
Spore chain on ISP 2	
Color of colony on ISP 5	
Substrate mycelium	White
Aerial mycelium	Green to olive-gray
Soluble pigment	None
Melanin pigment on ISP 1	None
Temperature range for growth	20–50°C
Optimum temperature for growth	25–38°C
Enzyme activity of	
arginine dihydrolase	+
lysine decarboxylase	+
tryptophane deaminase	+
ornithine decarboxylase	–
β-galactosidase	–
Decomposition of	
casein	+
gelatin	+
starch	+
lipid	+
urea	+
Production of	
indole	–
H_2S	–
nitrate	–
Carbon utilization	
D-glucose	–
D-mannitol	–
inositol	–
D-sorbitol	–
L-rhamnose	–

RESULTS AND DISCUSSION

Taxonomy

The strain M03033 was isolated from the sandy sediment collected from the intertidal zone off Daebu Island, Korea, and maintained on a yeast extract-malt extract agar containing 70% natural sea water. The colony of the strain grown at 30°C for 3 days was ivory to greenish ivory color, opaque, flat, regular, entire and matt type vegetative and white aerial mycelium, with spiral sporopore (*Spirales*). The strain was Gram positive and immobile. The optimum temperature of growth was at 25–38°C and the strain did not grow at 50°C or 20°C. A scanning electron microscopic picture of the strain showed the characteristic patterns of *Streptomyces*, straight chains tangled with numerous spores which are bead form with a smooth surface (Fig. 2).

The taxonomical properties of the strain are summarized in Table 1 and Table 2 [6, 11]. This strain produced arginine dihydrolase, lysine decarboxylase, and tryptophane deaminase, but ornithine decarboxylase and β-galactosidase were not produced. Chitin, gelatin, casein, urea, and starch were degraded and produced extracellular lipase. This strain could not utilize glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, or arabinose as a single carbon source.

A cellular fatty acid analysis revealed the presence of anteiso-pentadecanoic acid (a-C15:0) and iso-palmitic acid (i-C16:0) as the major fatty acids (Table 3). A cellular sugar analysis with TLC also showed the presence of arabinose only. Although an analysis of cell wall materials revealed only the presence of *meso*-form of diaminopimelic acid, the combination of the cultural, morphological, and physiological characteristics led us to assign the strain M03033 as a marine-derived actinomycete of the genus *Streptomyces*.

Production and Isolation

A slant culture of *Streptomyces* sp. M03033 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a SYP medium (starch 1%, yeast extract 0.4%, peptone 0.2%, K_2HPO_4 0.025%) in 70% aged sea water adjusted to pH 8 by 1 M Tris buffer, and incubated at 27°C for 4 days on a rotary shaker (150 rpm). Fermentation was carried out by a transfer of 20 ml of the seed culture to a 3-l Fernbach flask containing 0.8-l of the same medium and incubated for 7 days under similar condition.

Table 2. Chemotaxonomic characteristics of the strain M03033.

Characteristics	M03033
Cell wall type	IV
DAP	*meso*-DAP
Sugar	A
Phospholipid type	PI
Major fatty acid	*ante*-15:0 i-16:0

Table 3. Fatty acid composition of the strain M03033.

	Fatty acid	Content (%)
12:0	dodecanoic acid	2.8
14:0	tetradecanoic acid	4.5
i-14:0	iso-tetradecanoic acid	10.7
a-15:0	anteiso-pentadecanoic acid	25.6
i-16:0	isopalmitic acid	33.8
16:1	palmitoleic acid	14.6
i-17:0	iso heptadecanoic acid	8.6
	others	0.1

The pooled 45-l of culture broth was concentrated using a tangential filtering system (Minikross Lab. System, Micogen, U.S.A.) with a 0.5 μm poresize membrane. The medium contained in a wet mycelial cake was removed using a centrifuge for 10 min (8,500 rpm), and the residue (wet weight 1.27 kg) was extracted twice with MeOH (4-l×2). After removal of the solvent *in vacuo*, the residual brown gum (51.42 g) was suspended into MeOH (2-l) and filtered. The solvent was evaporated under reduced pressure, and the dark-brown residue (18.49 g) was subjected to C_{18} reversed-phase vacuum flash chromatography using sequential mixtures of MeOH and water as eluents (elution order: 50, 30, 20, 10% aqueous MeOH, and 100% MeOH). A bioactivity test revealed that the antifungal substances were contained in the 30% aqueous MeOH fraction (dry weight 1.39 g).

The filtered broth was subjected to adsorption chromatography. After loading the residue onto a Diaion

Table 4. ^{1}H and ^{13}C NMR assignments for *N*-formylantimycic acid methyl ester (**2**).

Position	^{1}H	^{13}C		FG-HMBC
1	4.76 (br d, J = 2.4 Hz)[a]	57.2	d	4, 10'
2	4.48 (dq, J = 2.1, 6.4 Hz)	67.8	d	
3	1.29 (d, J = 6.4 Hz)	20.2	q	1, 2
4		171.0	s	
OMe	3.81 (s)	52.9	q	4
1'	7.30 (dd, J = 8.2, 1.2 Hz)	120.3	d	3', 7', 10'
2'	6.87 (t, J = 8.2 Hz)	118.8	d	4', 9'
3'	8.48 (dd, J = 8.2, 1.2 Hz)	124.5	d	1', 7'
4'		126.9	s	
5'	7.97 (br s)			
6'	8.47 (br s)	159.0	d	4'
7'		150.0	s	
8'	12.62 (br s)			
9'		113.0	s	
10'		170.0	s	
11'	7.27 (br s)			

Assignments were aided by a combination of ^{1}H-^{1}H COSY, FG-HSQC, and FG-HMBC experiments. Numbering system employed that was adopted in a recent report [13].

[a]Proton resonance multiplicity and coupling constant in parenthesis.

HP20 column (5 cm×60 cm), the column was washed sequentially with distilled water (2-l), 50% aqueous MeOH (1-l), MeOH (1-l), and acetone (1-l). The biologically active acetone layer (0.47 g) was combined with the 30% aqueous MeOH fraction from the vacuum flash chromatography. The combined materials (1.86 g) were separated by a normal-phase HPLC (YMC silica column, 10×250 mm, 100% EtOAc) to yield antimycins complex (**1**) and *N*-formylantimycic acid methyl ester (**2**). The purification of these compounds was established by a reversed-phase HPLC (YMC ODS-A column, 35% aqueous MeOH) to produce 47.7 mg and 10.5 mg of **1** and **2**, respectively, as white amorphous solids.

Physicochemical Properties of 2

The molecular formula of this compound was established as $C_{13}H_{16}O_6N_2$ by a combined HRFAB-MS and ^{13}C NMR analyses. The UV data showed diagnostic peaks of antimycin chromophore at 326 and 229 nm [9, 14, 15]. IR absorptions at 3300 (broad) and 1735 cm^{-1} revealed the presence of hydroxyl (or amine) and ester functionality, respectively. Complex absorption bands at 1670, 1640 and 1545 cm^{-1} indicated the presence of amide groups. This compound was soluble in $CHCl_3$, EtOAc, and MeOH, and slightly soluble in hexane and water.

Structure Determination

The structures of **1**, antimycins A complex, were determined by combined NMR analysis and comparison of spectral data with those of authentic sample (Sigma). The NMR data of **2** showed characteristic signals of an aromatic ring; δ_H 6.8–8.5, δ_C 110–150 [13]. A ^1H-^1H COSY experiment revealed the presence of a 1,2,3-trisubstituted benzene moiety. The assignment of NMR signals at the benzene ring was established by combined FG (field gradient)-HSQC and FG-HMBC data (Table 4). The placement of a carbonyl and electronegative substituents, e.g. oxygen and nitrogen at C-9', C-7', and C-4', respectively, was made on the basis of the chemical shifts of aromatic carbons. The presence of a threonine methyl ester unit was secured by ^1H-^1H COSY and FG-HMBC experiments. A long-range correlation, observed between the protons at δ 3.81 and the carbon at δ 171.0, allowed the placement of a methoxy group at the carboxyl carbon. The peptide linkage between the threonine and benzoyl moiety was determined by a long-range correlation between H-1 at δ 4.76 and C-10' at δ 170.0. Consideration of the molecular formula as well as the chemical shift of a remaining carbon at δ 159.0 placed an aldehyde group at N-5', forming a foramido functionality.

The stereochemistry of threonine methyl ester was assigned by chemical degradation and GC analysis. Compound **2** was treated with 3 N HCl in MeOH at 80°C for 2 h and extracted with n-hexane. GC analysis of the hydrolysate under the same condition for fatty acid analysis (see Taxonomic Studies of M03033) gave a peak at retention time 17.68 min. D-Threonine methyl ester prepared from authentic sample by the same process gave a single peak at 17.63 min. Co-injection of the hydrolysate and authentic sample also gave a peak at 17.51 min. The stereochemistry of the threonine methyl ester of **2** was further supported by comparison of the specific rotation with that of an analog. The specific rotation of **2** {$[\alpha]_D^{25}$ +6.0° (*c* 0.29, MeOH)} was similar to the reported value of antimycic acid {$[\alpha]_D^{25}$ +14.9° (*c* 1.8, 3% HCl)} [1]. Thus, the D-configuration identical to that of antimycin was assigned for the threonine unit of *N*-formyl-antimycic acid methyl ester (**2**) [5].

A literature survey revealed that the degradation products of antimycin for structure determination included antimycic acid, *N*-formylantimycic acid, *N*-formylantimycic acid methyl ether methyl ester, and *N*-formylantimycic acid diester [4, 14, 15]. However, none of the efforts artificially converted antimycin to compound **2**. Furthermore, methylation only at C-4, but not at both C-4 and C-7 of the molecule, implied that **2** was a natural product.

Biological Activity

The antifungal activity was determined using the agar diffusion method against *Candida albicans*. The diameters of the clear zone for **1** was 1.9 cm at concentrations of 10 μg per disk. However, compound **2** was inactive at the same or higher concentrations (25 μg per disk).

Structure Activity Relationship of Antimycin

Antimycin is widely recognized as a potent antifungal agent. Biochemical studies revealed that the biological activity of this compound is mainly due to its inhibitory action on mitochondrial respiration [7]. That is, antimycin is a significant inhibitor of QH_2-cytochrome reductase which plays a key role in electron transfer between cytochromes *b* and c_1. The structure-activity relationship of the antimycins complex has attracted considerable attention. Studies showed that the lengths or types of side chains at the dilactone ring had little effect on the biological activity of antimycins [7]. On the contrary, the foramido and phenolic hydroxyl groups were found to be essential for the activity. Also, an important structural feature was the dilactone ring and the replacement of this portion by long-chain fatty acids, resulted in the significant loss of bioactivity. Our measurement of antifungal activity supported the previous results on the role of the dilactone ring on biochemical action. In addition, the lack of antifungal activity of **2** demonstrated that the maintenance of threonine was insignificant for activity. Therefore, the replacement of threonine with other building blocks may maintain the activity, which would help to prepare synthetic antifungal agents derived from antimycin.

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