YM-47515, a Novel Isonitrile Antibiotic from *Micromonospora echinospora* subsp. *echinospora*

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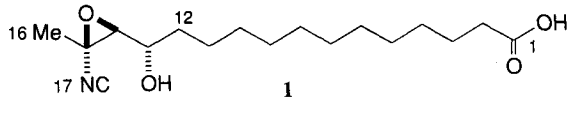
During the course of our screening for new antibiotics, *Micromonospora echinospora* subsp. *echinospora* Y-03559J was found to produce a novel isonitrile compound, YM-47515 (**1**) along with a probable degradation product **2**. The structure of **1** was assigned by spectroscopic analysis including 2D NMR and IR experiments. The relative stereochemistry of **1** was also proposed by comparison of spectral data with those of a closely related compound aerocyanidin (**3**). YM-47515 (**1**) showed promising antimicrobial activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA).

One of the most prominent groups of microorganisms yet to be examined as a potential source of bioactive compounds are the actinomycetes of the genus *Micromonospora*[1]. During our extensive investigation of *Micromonospora* species, we noticed that some aminoglycoside producing strains of this group, *e.g. M. purpurea*, *M. echinospora*, *M. inyonensis* and *M. olivoasterospora*, scarcely produced lipophilic antimicrobial substances. These findings prompted us to discover novel lipophilic antibiotics from aminoglycoside producers of *Micromonospora* species. Screening of 243 selected strains led us to find that a lipophilic portion of the fermentation extract of *M. echinospora* subsp. *echinospora* Y-03559J, which had been proved to produce gentamicin, showed intriguing antimicrobial activity. Herein, we report on the discovery of a novel antibiotic YM-47515 (**1**), whose structure featured an epoxide moiety bearing an isonitrile function. YM-47515 exhibited marked antimicrobial activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA). The producing organism, fermentation, isolation, structure elucidation and biological activity of **1** are described.



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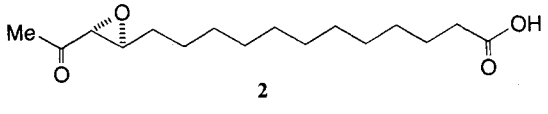

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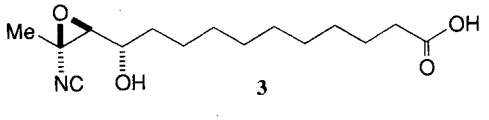

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Materials and Methods

Selection of Aminoglycoside-producing Strains

Aminoglycoside-producing strains of the genus *Micromonospora* listed in Table 1 (243 strains in total) were selected from our culture collections using the conventional methods of analyzing their cultural extracts as follows: (a) detection of antimicrobial activty against aminoglycoside-resistant and -supersensitive bacterial strains and (b) analysis of chemical components by TLC

Table 1. *Micromonospora* strains selected as aminoglycoside producers.

Aminoglycoside	Number of producing strains
Neomycin	5
Gentamicin	103
Fortimicin	100
Verdamicin	3
Sisomicin	32
Total	243

(colorimetric reactions and Rf values) and/or mass spectral measurements.

Screening for Lipophilic Antibiotics

For screening, cultures were grown at 28 and 32°C for four to twelve days using 36 different media for each strain. At harvest, the culture broth was mixed with an equal volume of EtOAc. The EtOAc layer was separated and concentrated to dryness, and the residue was dissolved in DMSO/H_2O (7:3). The DMSO sample was examined for antimicrobial activity against a panel of Gram-positive and Gram-negative bacteria.

Taxonomic Studies

Morphological, cultural and physiological properties were examined according to the methods described by SHIRLING and GOTTLIEB[2]. Incubation for cultural properties was employed at 28°C for 14 to 21 days. Chemical composition of the cell was determined using the methods of LECHEVALIER and LECHEVALIER[3], and BECKER *et al.*[4]. Color names were taken from the Guide to Color Standard of Nihon Shikisai Co., Ltd.

Fermentation of Strain Y-03559J

A loopful of the microorganism grown on an agar slant was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 1.0%, potato starch 2.0%, yeast extract 0.5%, Polypepton (Nihon Pharmaceutical Co., Ltd.) 0.5% and $CaCO_3$ 0.4%. The pH of the medium was adjusted to 7.0 before sterilization. The seed culture was incubated at 28°C for 48 hours on a rotary shaker at 220 rpm, and then transferred into a 30-liter jar fermenter containing 18 liters of the same medium. After incubation at 28°C for 72 hours, the second seed culture was used to inoculate a 300-liter jar fermenter filled with 240 liters of a production medium consisting of soluble starch 3.0%, casein 0.06%, K_2HPO_4 0.4%, KNO_3 0.4%, NaCl 0.4%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCO_3$ 0.004% and $FeSO_4 \cdot 7H_2O$ 0.002%. The pH of the medium was adjusted to 7.0 before sterilization. The fermentation was run for 72 hours at 28°C with an agitation rate of 200 rpm and aeration of 1 vvm.

General Procedures

^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-A500 NMR spectrometer. An infrared spectrum was recorded on a Hitachi 260-50 infrared spectrometer. An optical rotation was determined by a JASCO DIP-370 digital polarimeter. Mass spectra were measured on a VG ZAB-VSE mass spectrometer.

Results and Discussion

Screening Results

As a result of screening, only a Y-03559J strain out of 243 aminoglycoside producers was found to produce a lipophilic antibiotic. This strain also produced gentamicin.

Taxonomy of Strain Y-03559J

Strain Y-03559J was isolated from a soil sample collected in Chichibu-shi, Saitama, Japan. The vegetative mycelia were well developed and branched. No aerial mycelium was formed. Non-motile spores were borne singly on monopodial sporophores. The spores were spherical and measured 0.6~1.0 μm with a spiny surface. The color of spores was dark brown to black under a phase contrast microscope. Sclerotic granules and sporangia were not observed. This strain grew well on oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4) with the reverse color of brownish purple, whereas the growth on tyrosine agar (ISP 7) and glucose-asparagine agar was very poor. The color of the vegetative mycelium was orange to purple. No soluble pigment was formed on any media. The utilized carbon sources were L-arabinose, D-xylose, maltose, sucrose and starch. The whole cell hydrolysate contained *meso*- and 3-OH-diaminopimelic acid. The diagnostic sugars were xylose and arabinose, indicating that the culture had a type IID cell wall. The predominant isoprenoid quinone was MK-10 (H_4).

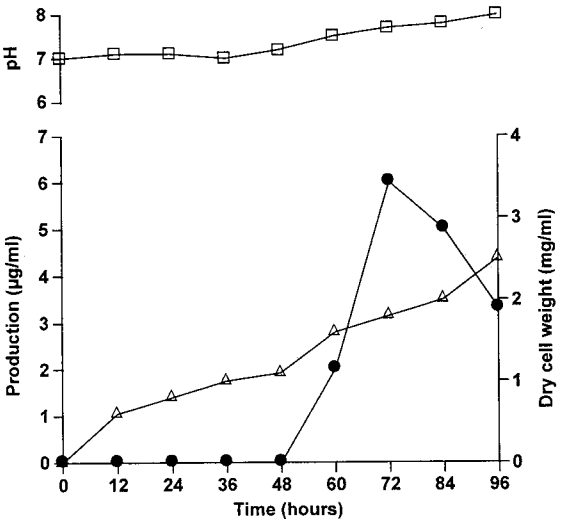
Based on morphological and chemotaxonomic characteristics, the strain could be assigned to the genus *Micromonospora*. In addition, the cultural and physiological properties, and carbon utilization agreed well with the published description of *Micromonospora echinospora* subsp. *echinospora* Luedemann and Brodsky[5]. Thus, the strain was designated *Micromonospora echinospora* subsp. *echinospora* Y-03559J. The producing culture has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with an accession number of FERM P-13677.

Production and Isolation

A typical fermentation profile of strain Y-03559J is shown in Fig. 1. The production of YM-47515 started after 48 hours of fermentation, reached a maximum at 72 hours, and then dropped.

Fig. 1. Time course of YM-47515 (**1**) production in a 300-liter tank fermenter.

□ pH, ● YM-47515 production, △ dry cell weight.



A total of 240 liters of the culture broth was harvested after 72 hours of fermentation, and was then filtered. The filtrate was passed through a column of Diaion HP-20 resin (15 liters). After washing the column with water and acetone/water (2:8), active materials were eluted with acetone/water (8:2). Antimicrobial activity was monitored by a paper-disk assay on agar plates seeded with *Staphylococcus aureus* FDA 209P. The acetone/water (8:2) eluate (20 liters) was concentrated *in vacuo* to 7 liters, adjusted to pH 5.5 with 4 N HCl, and extracted with EtOAc twice to give an oily residue. The residue was distributed in hexane/toluene/MeOH/water (3:3:4:2), and the lower layer was evaporated to remove organic solvents. The resultant aqueous solution was extracted with EtOAc twice. The extract was successively fractionated by ODS flash chromatography on Cosmosil 140C_{18}-OPN (Nacalai Tesque Co., Ltd.) and on Cosmosil 75C_{18}-OPN with increasing amounts of MeOH in water. The fractions eluted with MeOH/water (7:3 and 9:1) were combined and subjected to centrifugal partition chromatography with hexane/EtOAc/MeOH/water (1:1:1:1) system using the upper layer as the mobile phase. The active fraction was repeatedly separated by ODS column chromatography using Cosmosil 140C_{18}-OPN with MeCN/water (55:45) and Cosmosil 75C_{18}-PREP with MeCN/water (1:1). Final purification was achieved by centrifugal partition chromatography with hexane/CH_2Cl_2/MeOH/water (5:1:1:1) system using the lower layer as the mobile phase to give 3.2 mg of YM-47515 (**1**).

Table 2. Physico-chemical properties of YM-47515 (**1**).

Appearance	Colorless gum
Molecular weight	311
Molecular formula	$C_{17}H_{29}NO_4$
HRFAB-MS (*m/z*)	
Found:	334.1990 $(M+Na)^+$
Calcd:	334.1994
$[\alpha]_D^{25}$	−23.8° (*c* 0.08, MeOH)
IR (film) cm^{-1}	3350, 2940, 2860, 2140, 1810, 1720, 1470

Careful examination of the fractions from the above ODS column also led to the isolation of **2**, which was most likely to be a degradation product of **1**. The relevant fractions were combined and then applied to ODS HPLC on Cosmosil 5C_{18}-AR with MeCN/water (45:55) to yield 2.3 mg of **2**.

Structure Determination

The physico-chemical properties of YM-47515 (**1**) are listed in Table 2. **1** was considerably labile to acid and base, and it was observed during the NMR measurements in $CDCl_3$ that **1** was gradually decomposed probably due to a trace amount of hydrochloric acid in the solvent. Therefore, NMR studies were performed using CD_3OD. YM-47515 (**1**) had a molecular formula of $C_{17}H_{29}NO_4$, which was established by high-resolution FAB-MS and NMR data (Table 3). The ^{13}C NMR spectrum exhibited 16 out of 17 carbons. The following types of these carbons were ascribed from the ^1H NMR and DEPT spectra: a methyl, eleven methylenes, two oxygenated methines, a quaternary carbon and an acid/ester carbonyl. Although a remaining carbon signal was missing, the IR absorption at 2140 cm^{-1} indicated the presence of an isonitrile functionality, which was supposed to cause crucial broadening of this signal associated with coupling to an adjacent quaternary nitrogen. In the FAB-MS experiment, **1** gave a monomethyl ester ion upon reaction with diazomethane, suggesting that it had a free carboxyl group. In addition, the molecular formula indicated the presence of a free hydroxyl. Taking into account four degrees of unsaturation, three of which were assignable to a carboxyl and an isonitrile group, **1** had a ring moiety.

The ^1H NMR analysis showed a singlet methyl (δ 1.73), an α-to-carbonyl methylene (δ 2.25), two methines (δ 2.87 and 3.50), and an aliphatic envelope (δ 1.30~1.68) indicating the presence of a long methylene chain,

Table 3. ^1H and ^{13}C NMR data of YM-47515 (**1**) and **2** in CD_3OD.

No.	1		2	
	^{13}C	^1H	^{13}C	^1H
1	178.7 (s)		179.0 (s)	
2	35.8 (t)	2.25 (t, 7.3)	36.0 (t)	2.24 (t, 7.7)
3	26.5 (t)	1.59 (m)	26.5 (t)	1.59 (m)
4	[a]	[b]	30.5 (t)	1.30 (m)
5	[a]	[b]	[d]	[e]
|	[a]	[b]	[d]	[e]
10	[a]	[b]	[d]	[e]
11	25.9 (t)	1.52 (m)	[d]	[e]
12	35.8 (t)	1.70 (m), 1.62 (m)	32.9 (t)	1.57 (m)
13	70.1 (d)	3.50 (ddd, 8.5, 8.5, 4.3)	59.5 (d)	3.11 (td, 5.5, 1.8)
14	66.1 (d)	2.87 (d, 8.5)	60.9 (d)	3.23 (d, 1.8)
15	67.1 (s)		208.0 (s)	
16	22.3 (q)	1.73 (s)	24.8 (q)	2.06 (s)
17	[c]			

[a] Methylene signals were observed between δ 30.7 and 30.4.
[b] Signals were overlapped between δ 1.30 and 1.68.
[c] Not observed.
[d] Methylene signals were observed between δ 30.8 and 26.9.
[e] Signals were overlapped between δ 1.28 and 1.67.

which was confirmed by DEPT data. The combination of HMQC and DQF-COSY spectra allowed construction of a structural fragment C12-C14. It was also shown that $H_2$12 were coupled to a methylene envelope. Further structural verification was done by interpretation of the HMBC experiment. The singlet methyl protons $H_3$16 (δ 1.73) gave cross peaks to C14 and C15, whereas H13 (δ 3.50) was correlated to C11, C12, C14 and C15. These HMBC correlations established the connectivity from C11 to C16 containing three contiguous oxygenated carbons (C13-C15). Judging from the chemical shifts of H13 (δ 3.50) and H14 (δ 2.87), it was evident that a hydroxyl group was located at C13, and C14 was participating in the epoxide formation along with C15, which was supported by the $^1J_{CH}$ values of 141 Hz for C13 and 178 Hz for C14. Thus, **1** had a trisubstituted epoxide at the left terminus. The partial structure of the right-hand portion (C1-C3) containing a carboxyl terminal was straightforward by the HMBC correlations ($H_2$2/C1, C3; $H_2$3/C1). In the DQF-COSY spectrum, $H_2$2 was coupled to a methylene envelope as well as $H_2$12, which assembled the above partial structures (C1-C3 and C11-C16) through an unbranched methylene chain. The remaining isonitrile functionality was unequivocally placed at C15, which was in accordance with the carbon chemical shift of C15 (δ 67.1)[6].

The ^1H and ^{13}C NMR data of **2** were very similar to those of **1**. The high-resolution FAB-MS analysis of **2** gave a molecular formula of $C_{16}H_{28}O_4$, which corresponded to the loss of an isonitrile unit from **1**. The ^{13}C NMR spectrum indicated the presence of an additional carbonyl, which was assignable to a saturated ketone based on its chemical shift (δ 208.0). Simultaneous analysis of DQF-COSY, HMQC and HMBC data led to unambiguous assignment of the gross structure **2**, possessing a disubstituted epoxide adjacent to a methyl ketone. The geometry of the epoxide ring was revealed to be *trans* based on the vicinal coupling constant of 1.8 Hz for H13-H14, which was supported by NOESY cross peaks between $H_2$12 and H14, and between H13 and $H_3$16.

YM-47515 (**1**) happened to be an elongated analogue of aerocyanidin (**3**)[7], which had been isolated from a bacterial strain of *Chromobacterium violaceum*. Furthermore, compound **2** corresponded to the degradation product of **3** resulting from treatment with base, allowing us to assume that YM-47515 (**1**) was decomposed to give **2** during the purification process. The relative stereochemistry of **1** was tentatively assigned to be the same as that of aerocyanidin (**3**), since the ^1H NMR chemical shifts of **1** and **3** were very similar, and compound **2** and the degradation product of **3** had the same relative configuration.

YM-47515 (**1**) and aerocyanidin (**3**) belonged to a rather small class of natural products characterized by an epoxide bearing an isonitrile group. It is noteworthy

Table 4. Antimicrobial activity of YM-47515 (**1**).

Organism	Zone diameter (mm)
Staphylococcus aureus FDA 209P	31
Staphylococcus aureus (MRSA-1)	30
Staphylococcus aureus (MRSA-2)	33
Staphylococcus aureus (MRSA-3)	29
Staphylococcus aureus (MRSA-4)	33
Staphylococcus epidermidis IID 866	36
Bacillus subtilis ATCC 6633	17
Mycobacterium smegmatis ATCC 607	0
Micrococcus sp.	0
Escherichia coli K-12	17
Proteus mirabilis IFMOM-9	0
Pseudomonas aeruginosa NCTC 10490	0
Candida albicans ATCC 10236	0

Inhibitory zones were measured at 100 μg/ml of YM-47515 after overnight incubation at 28°C for bacteria and 32°C for fungi.

that **1** and **3**, both of which had an almost identical carbon skeleton, were isolated from taxonomically different sources of an actinomycete and an eubacterium, respectively.

Biological Activity

Antimicrobial activity of YM-47515 (**1**) was evaluated by measuring the diameter of an inhibitory zone at the concentration of 100 μg/ml in the paper-disk method (Table 4). Due to a limited amount of the sample, exact MIC values for **1** was difficult to determine.

1 exhibited excellent activity against *Staphylococcus aureus* strains including MRSA, and little or no activity against Gram-negative bacteria or fungi.

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