Discovery of Methyl-2-(2′-hydroxyphenyl)-2-oxazoline-4-carboxylate as a Secondary Metabolite from *Actinomadura* sp.

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In our screening programs for novel antibacterial antibiotics of microbial origin, we found an oxazoline-type antibiotic in the cultural broth of the producing organism, designated MJ502-77F8 and belonging to a rare Actinomycetes group. In this paper, we report on the preliminary taxonomical characteristics of the producing strain, production, isolation, structural elucidation, and biological activity of this antibiotic, herein referred to as antibiotic **1**.

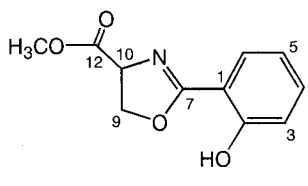
The producing strain MJ502-77F8 was isolated from a soil sample collected in Setagaya-ku, Tokyo, Japan. The taxonomy was assigned by following the procedure of the International Streptomyces Projects (ISP), as recommended by SHIRLING and GOTTLIEB[1]. The strain exhibited the following cultural characteristics during incubation for 3 weeks at 27°C in various media. It formed well-branched substrate mycelium and short aerial mycelium. The color of the aerial mass was light grayish blue to grayish blue, and that of the vegetative growth was pale yellow to strong reddish orange in ISP media No. 2 and No. 4. Typical melanoid and soluble pigments were not produced. Microscopic observation showed the mature sporophores to form hooked spirales and short spore chains having more than 10 spores per chain, and the spores to be oval with a warty surface. Neither sclerotic granules and sporangia nor flagellated spores were observed. This strain utilized L-arabinose, D-xylose, D-glucose, L-rhamnose, sucrose, soluble starch and dextrin for growth in ISP medium No. 9 without $CuSO_4$, but could not grow on D-fructose, raffinose, inositol, D-mannitol, D-galactose, salicin, or maltose. Utilization of glycerin was doubtful.

The whole cell hydrolysis of strain MJ502-77F8 contained *meso*-diaminopimelic acid and madurose, and analysis of the whole-cell phospholipids revealed the presence of diphosphatidylglycerol and phosphatidylinositol, as determined by the method of LECHEVALIER *et al.*[2]. Therefore, this strain was classified as having cell wall sugar pattern B and phospholipid type PI. Mycolic acids were not detected. The predominant isoprenoid quinone consisted of hexa- and octahydrogenated menaquinone with nine isoprene units, MK-9(H_6) and MK-9(H_8). Based on the morphological and chemotaxonomical results, the above characteristics of strain MJ502-77F8 were in good accordance with those of the genus *Actinomadura*[3], although the species was not assigned. The strain has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukuba, Japan, under the name *Actinomadura* sp. MJ502-77F8 and the accession number FERM BP-5184.

Antibiotic **1** was produced by fermentation of *Actinomadura* sp. MJ502-77F8. A loopful of agar slant culture of the producing strain was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium containing glucose, 0.1%; tryptose (Difco), 0.5%; soluble starch, 2.4%; meat extract, 0.3%; yeast extract, 0.5%; $CaCO_3$, 0.2% (pH 7.0 before autoclaving). The seed culture was incubated at 27°C for 4 days operating on a rotary shaker at 220 rpm. Twelve milliliters of the seed culture thus obtained were inoculated into each of several 500-ml Erlenmeyer flasks containing 200 ml of a producing medium having the same composition as the seed medium. The fermentation was carried out at 27°C for 7 days operating on a rotary shaker. The progress of production in culture was monitored by the agar diffusion method with *Staphylococcus aureus* 209P as the tested organism.

Antibiotic **1** was purified by the following procedures: The culture broth (60 liters) was centrifuged at 2,800 rpm for 10 minutes to separate the mycelial cake and then filtered. The broth filtrate was adjusted to pH 4 with dil. HCl, and extracted 3 times with ethyl acetate. In addition, the concentrate obtained by two extractions of the mycelial cake with methanol was adjusted to pH 4 and extracted 3 times with ethyl acetate. The combined ethyl

Fig. 1. Structure of antibiotic **1**.



acetate extracts were then dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to yield the crude extract (14.8 g). The crude extract, after having been dissolved in a small amount of $CHCl_3$-MeOH-AcOEt-HCOOH-H_2O (180:20:50:1:1), was applied to a silica gel column (36×8 cm, i.d., Merck), and then the active fractions were eluted with the same solvent to separate the other antibacterial components. The active fractions that eluted early were neutralized with dil. NH_4OH and evaporated to dryness (3.6 g). After that, this sample was further purified by silica gel column (30×4.2 cm, i.d.) chromatography with $CHCl_3$ as the eluent solvent. The corresponding active fractions were combined and evaporated to give the purified oily material (203 mg). Thin-layer of chromatography on silica gel $60F_{254}$ (Merck) with $CHCl_3$ or AcOEt-hexane (2:8) as the developing solvent gave a single spot of Rf=0.7 or 0.5, respectively, that showed inhibitory activity in the bioassay with *S. aureus*.

Physico-chemical properties of antibiotic **1** were as follows: EI-MS *m/z* 221 $(M)^+$; UV $\lambda_{max}^{CHCl_3}$ nm 238, 247, 253sh, 306, $[\alpha]_D^{20}$ −50.1°C (*c* 1.46, $CHCl_3$/MeOH), IR (film) cm^{-1} 1746, 1641, 1616, 1493, 1370, 1261, 1233, 758, ^{1}H-NMR (400 MHz in $CDCl_3$, 30°C) 11.66 (1H, s, 2-OH), 7.01 (1H, dd, *J*=8.5, 1.5 Hz, 3-H), 7.38 (1H, ddd, *J*=8.5, 7.5, 1.5 Hz, 4-H), 6.87 (1H, ddd, *J*=8.0, 7.5, 1.5 Hz, 5-H), δ 7.65 (1H, dd, *J*=8.0, 1.5 Hz, 6-H), 4.67 (1H, dd, *J*=9.0, 7.5 Hz, 9-H), 4.56 (1H, dd, *J*=10.5, 9.0 Hz, 9-H), 4.97 (1H, dd, *J*=10.5, 7.5 Hz, 10-H), 3.80 (3H, s. 12-OCH_3), ^{13}C-NMR (100 MHz in $CDCl_3$, 30°C) δ 110.00 (s, 1-C), 159.89 (s, 2-C), 116.86 (d, 3-C), 133.94 (d, 4-C), 118.73 (d, 5-C), 128.30 (d, 6-C), 167.49 (s, 7-C), 68.84 (t, 9-C), 67.14 (d, 10-C), 170.88 (s, 12-C), 52.69 (q, OCH_3). On the basis of these analytical findings, the chemical structure of **1** was deduced to be methyl 2-(2′-hydroxyphenyl)-2-oxazoline-4-carboxylate, as shown in Fig. 1. In addition, the structure of **1** was confirmed by ^{1}H-^{13}C long range couplings observed in an HMBC experiment. The characteristic UV spectrum of **1** was similar to that of mycobactins, which were isolated as iron-chelating growth factors from *Mycobacterium* species, as reviewed by SNOW[4]. The structure of mycobactins consists of mycobactic acid unit as the parent molecule possessing the phenyloxazoline ring system and cobactin unit. Among the mycobactin group of compounds, the mycobactic acid moiety can differ in having or not a methyl group at position 6 in the benzene ring and at position 9 in the oxazoline ring (referred to Fig. 1). In synthetic studies on the chromophore of mycobactins, hydroxyphenyloxazoline and its ester derivatives related to **1** were prepared by BLACK *et al.*[5]. The physico-chemical properties of **1** agree fully with those reported for synthetic compound, methyl 2-(2′-hydroxyphenyl)-2-oxazoline-4-carboxylate, in terms of UV, IR, MS, and NMR spectra. Very recently, MURAKAMI *et al.*[6] reported a novel mycobactin group antibiotic, formobactin, that they isolated from *Nocardia* sp. No-20 and demonstrated to be a free radical-scavenging and neuronal cell-protecting substance. As partial structure of formobactin, astezoidic acid moiety, which is 2-hydroxybenzene attached to an 3-methyloxazole ring moiety, was preserved.

Table 1. Antimicrobial activity of antibiotic **1**.

Test organisms	MIC (μg/ml)
Staphylococcus aureus FDA 209P	25
S. aureus Smith	25
S. aureus 70 (MRSA)	25
S. aureus 92-1192 (MRSA)	25
S. epidermidis ATCC 12228	6.25
Micrococcus luteus ATCC 9341	>100
Bacillus subtilis ATCC 6633	25
B. subtilis (rec+)	25
B. subtilis (rec-)	6.25
B. cereus IFO 3001	>100
Escherichia coli NIHJ	12.5
E. coli K-12	>100
Proteus vulgaris IID OX-19	>100
Morganella morganii IFO 3848	>100
Klebsiella pneumoniae ATCC 29665	>100
Serratia marcescens IFO 12648	>100
Salmonella typhymurium G-46	>100
Pseudomonas aeruginosa NCTC 10490	>100

MRSA: Methicillin-resistant *S. aureus*.
MIC of methicillin against MRSAs 70 and 92-1192 was 50 μg/ml.

The antimicrobial activity of antibiotic **1** was determined on Mueller-Hinton agar (Difco) by the serial agar dilution method. Approximately 10^4 cfu per spot were inoculated onto each of several agar plates. The determination of MIC, the lowest concentration of antibiotic that inhibits completely the visible growth after incubation for 18 hours at 37°C, showed **1** to have antimicrobial activity against some Gram-positive bacteria including highly methicillin-resistant *Staphylococcus aureus* (MRSA), but no activity against most Gram-negative bacteria except for *Escherichia coli* NIHJ, as shown in Table 1. Growth inhibitory activity against *Bacillus subtilis* exhibited at the concentration of 50 μg/ml of **1** on agar plate was almost completely antagonized by the

addition of 50 mM $FeCl_3$. In addition, **1** showed cytotoxic activity with IC_{50} concentration of 0.3 μg/ml when human nasopharyngeal carcinoma KB cells were exposed to it for 3 days at 37°C. The cytotoxicity test of antibiotic **1** was done as follows: KB cells (2×10^3 cells/well) were seeded into a 96-well multiplate containing EAGLE's MEM supplemented with 10% calf serum. After one day of incubation, **1**, dissolved in dimethylsulfoxide was added to the wells. After 3 days, cell numbers were estimated by the dye-uptake method[7]. However, those biological activities of **1** as mentioned above were not reported in synthetic compounds prepared by BLACK *et al.*

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