Mer-A2026A and B, Novel Piericidins with Vasodilating Effect

I. Producing Organism, Fermentation, Isolation and Biological Properties

KAICHIRO KOMINATO*, YOSHIO WATANABE, SHIN-ICHI HIRANO, TOMOYUKI KIOKA, TADASHI TERASAWA, TAKEO YOSHIOKA, KAZUHIKO OKAMURA and HIROSHI TONE

Central Research Laboratories, Mercian Corporation, 9-1 Johnan 4 Chome, Fujisawa, Kanagawa 251, Japan

(Received for publication August 17, 1994)

A strain of *Streptomyces* was found to produce new piericidins. The compounds were purified and separated into two substances named Mer-A2026A and B.

These new piericidins exhibited vasodilating and depressor activities.

In the course of our screening program for new physiologically active substances, a streptomycete, strain Me2108, identified as *Streptomyces pactum* was found to produce new substances which exhibit vasodilating activities.

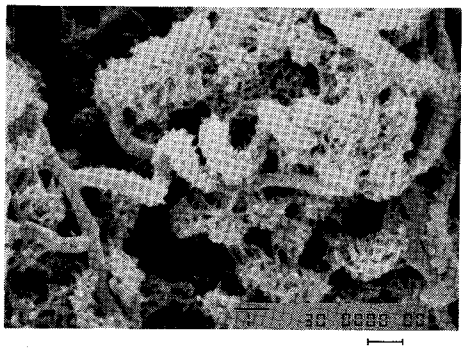
In this report we provide the taxonomy of producing strain, and the fermentation, isolation, and biological properties of Mer-A2026A and B.

Taxonomy of the Producing Strain

Strain Me2108 was isolated from a soil sample collected in the riverside of Hikichi river, Fujisawa City, Kanagawa Prefecture, Japan in 1989 (hereinafter referred to as *Streptomyces pactum* Me2108).

As morphological characteristics, the aerial hypha of the cultured strain is well branched and the top of the aerial hypha is closed spiral (Fig. 1). After growing up, it becomes divided and forms a spiral chain of conidia. The size of spherical or elliptical conidium is about $1.0 \times 1.0 \sim 1.2\,\mu m$. The surface of the conidium is hairy with a lot of fine hair. No flagellum is observed.

Fig. 1. Aerial mycelium of strain Me2108. Oatmeal agar, 14 days at 28°C, (×28,000).



The strain has the following cultural characteristics when grown on media as described below at 30°C. The color of the surface of colony is indicated according to the symbols described in Color Harmony Manual[1]. On yeast extract-malt extract agar, good growth with abundant aerial hyphae and conidia are observed. The color of the aerial mass of colony is faint gray reddish brown, Ashes (5fe). Melanoid pigment and soluble pigment are not observed. On tryptone yeast extract agar, moderate growth with slight aerial hyphae formation is observed. The color of the surface of colony is white (a). Very small amount of or almost no conidium is observed.

The carbon source utilizing pattern according to Pridham-Gottlieb medium[2] are summarized in Table 1.

L,L-Diaminopimelic acid is observed as one of the components of cell wall when cell wall is analyzed by cellulose thin layer chromatography after hydrolyzation. No particular pattern of sugar is observed. The type of cell wall is suggested to be Type I[3].

From the foregoing taxonomic characteristics, it is apparent that the strain Me2108 belongs to *Streptomyces* genus and identical with those of *Streptomyces pactum*

Table 1. Carbon utilization of strain Me2108.

Carbon source	Growth
L-Arabinose	−
D-Xylose	−
D-Glucose	+
D-Fructose	−
Sucrose	−
Inositol	−
L-Rhamnose	−
Raffinose	−
D-Mannose	−

+; Positive, −; negative.

published in International Streptomyces Project (ISP)[4,5], except that the utilization of fructose and pigment production are described as being ± in the ISP publication. Therefore strain Me2108 was identified as *Streptomyces pactum* and designated *Streptomyces pactum* Me2108.

Fermentation

A well-sporulated slant of *S. pactum* Me2108 was inoculated into 500-ml flasks each containing 100 ml of a medium composed of glycerol 2.0%, glucose 2.0%, soybean meal 2.0%, yeast extract 0.5%, sodium chloride 0.25%, calcium carbonate 0.32%, and 0.2% of metal salt solution containing 0.25% copper sulfate, 0.25% manganase chloride, and 0.25% zinc sulfate. The pH of the medium was adjusted to pH 7.4 before sterilization. The flasks were incubated on a rotary shaker at 28°C for 3 days.

The fermentation was carried out in a 30-liter jar fermentor containing 15 liters of the same as the vegetative medium except that potato starch 2.0% was used instead of glycerol 2.0%. A defoaming agent (0.05%) was added to the culture medium for jar fermentation. After inoculation of 200 ml of a seed culture the fermentation was conducted at 28°C for 4 days under aeration of 1 vvm and agitation of 300 rpm.

A typical time course of fermentation in a 30-liter jar fermentor is shown in Fig. 2. The amount of Mer-A2026B and A reached a maximum after 70 hours and 90 hours fermentation, respectively.

Isolation and Purification

The isolation and purification procedure of Mer-A2026A and B is shown in Fig. 3.

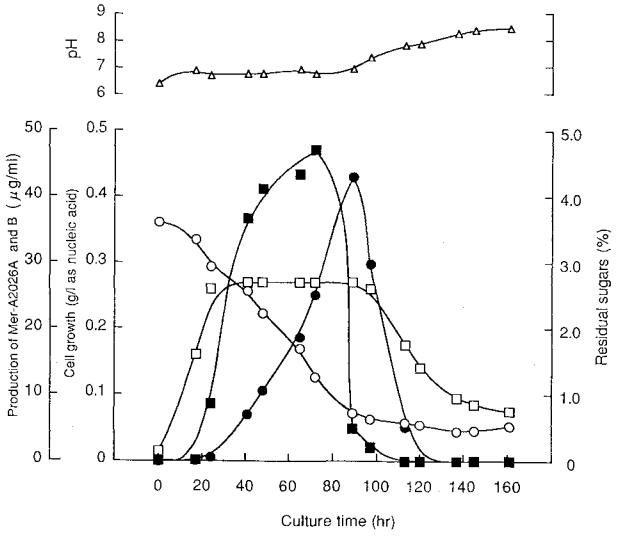
The culture broth was centrifuged to separate the mycelia from the broth. The supernatant was extracted three times with a half volume of *n*-butanol. The butanol extract was concentrated under reduced pressure to dryness.

The crude product was dissolved in methanol, and then a same volume of ether was added to precipitate impurities which were then removed by filtration. The filtrate was concentrated and the resulting residue was then dissolved in methanol, applied on a column of Sephadex LH-20 (400 ml) and fractions containing active substances were collected using methanol as an eluent. The fractions were concentrated to give residue containing active substances.

The residue was dissolved in chloroform - methanol (50 : 1), applied to a column of 250 ml silica gel (Kieselgel 60, Merck) and eluted with the solvent composed of chloroform and methanol.

Fig. 2. Time course of production of Mer-A2026A and B by *Streptomyces pactum* Me2108.

Mer-A2026A (●) and Mer-A2026B (■) determined by the HPLC method, residual sugars (○) determined by the phenol-sulfuric acid method, cell growth (□) monitored with crude nucleic acid extraction, pH of culture broth (△).



The active fraction was concentrated, dissolved in toluene - acetone (5 : 1), applied on a column of 50 ml silica gel (Kieselgel 60, Merck) and eluted with the solvent composed of toluene and acetone.

The active fraction was concentrated separately and applied to a gel filtration using Sephadex LH-20 (100 ml) to afford Mer-A2026A (25.5 mg) and Mer-A2026B (5.0 mg), respectively.

Vasodilating Effect

The thoracic aortae were removed from male Sprague-Dawley rats, 250 ~ 300 g, and dissected free from fat and connective tissues. Helically cut strips, approximately 3 mm wide and 10 mm length, were prepared. The endothelial layers were removed by gently rubbing the intimal surface with a finger moistened with physiological saline solution. Muscle strips were maintained in physiological salt solution (PSS) and bubbled with 95% O_2 ~ 5% CO_2 at 37°C. The composition of PSS was as follows (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 2.5, $MgSO_4$ 1.0, $NaHCO_3$ 23.8 and glucose 5.5. Hyper isosmotic 65.4 K^+ (H-K) solution was made by replacing 60 mM NaCl in the above solution with equimolar KCl. The muscle strips were suspended in an organ bath (10 ml) under the resting tention of 0.5 g and contraction was recorded isometrically with a force transducer (TB-611T, Nihon Kohden).

Mer-A2026A and Mer-A2026B were dissolved in dimethylsulfoxide (DMSO), and these agents were

Fig. 3. Flow diagram for the isolation and purification of Mer-A2026A and B.

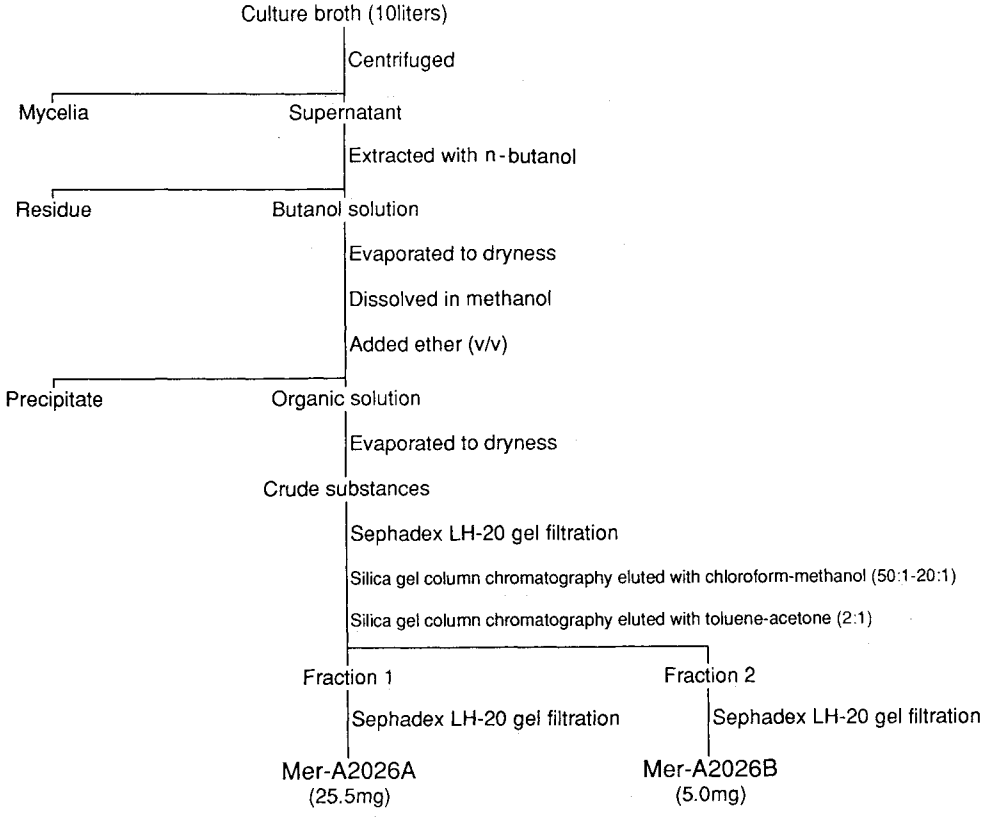
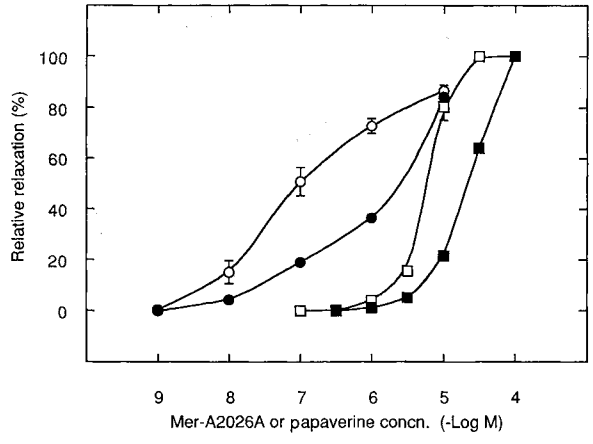


Fig. 4. Concentration-relaxation curves for Mer-A2026A in the rat thoracic aorta.

Mer-A2026A (○) and papaverine (□) in the aorta precontracted with 10^{-7} M norepinephrine (NE), Mer-A2026A (●) and papaverine (■) in the aorta precontracted with hyper isosmotic 65.4 mM K^+ (H-K). Data points are represent the mean ± S.E. of 4 to 8 experiments.



cumulatively applied in the organ bath during the sustained contraction induced by 10^{-7} M norepinephrine (NE) or H-K. Final concentration of DMSO was 0.4% or less.

The concentration-relaxation curves for Mer-A2026A and EC_{50} values in vasodilating activities of Mer-A2026A and B are shown in Fig. 4 and Table 2, respectively.

Mer-A2026A ($10^{-8} \sim 10^{-5}$ M) relax the aortae pre-

Table 2. Vasodilating effects of Mer-A2026A and B in rat aorta.

Compound	EC_{50} (M)	
	NE	H-K
Mer-A2026A	2.0×10^{-7}	1.0×10^{-6}
Mer-A2026B	7.7×10^{-7}	4.0×10^{-7}
Papaverine	4.7×10^{-6}	1.7×10^{-5}

Each value is calculated by the mean value of 4 to 8 experiments.
NE; 10^{-7} M norepinephrine, H-K; hyper isosmotic 65.4 mM K^+.

contracted with NE than in those with H-K. The EC_{50} values of Mer-A2026A in the NE- and H-K-induced contraction were 2×10^{-7} M and 10^{-6} M, respectively. Mer-A2026B ($10^{-8} \sim 10^{-5}$ M) showed similar effects in the concentration-relaxation curves (data not shown). However, Mer-A2026B did not show the difference between in the effects on NE and in those on H-K. Also, the EC_{50} values of this agent in the NE- and H-K-induced contraction were 7.7×10^{-7} M and 4×10^{-7} M, respectively. The relative potency of both agents were greater than that of papaverine, a standard vasodilating drug.

Depressor Effect

Male spontaneously hypertensive rats (SHR), 350 ~ 400 g, 25 ~ 31 weeks old, were anesthetized with 45 mg/kg of sodium pentobarbital injected intraperitoneally. For the measurement of systolic, diastolic, and mean blood

Table 3. Depressor effect of Mer-A2026A in spontaneously hypertensive rats.

Compound	Dose (mg/kg, i.v.)	Depression (mmHg)	Duration time (minute)
Mer-A2026A	0.01	3.6± 2.1	0.2± 0.1
	0.03	25.0± 4.3	9.4± 2.8
	0.1	47.7±14.2	24.5± 9.9
Papaverine	1.0	52.6± 6.6	2.2± 0.2

Each value indicates the mean±S.E. of 3 to 5 experiments.

pressures, a cannula was inserted into the left carotid artery and connected to a multipurpose polygraph (RM-6000, Nihon Kohden) through a pressure transducer (MPU-0.5, Nihon Kohden).

Mer-A2026A was dissolved in 0.9% physiological saline solution contained less than 5% of DMSO, and injected into the femoral vein of SHR through a polyethylene tube.

The depression in mean blood pressure and its duration time caused by Mer-A2026A are shown in Table 3.

Mer-A2026A (0.01~0.1 mg/kg) produced a dose-dependent and marked decrease in the mean blood pressure. Also, Mer-A2026A at doses of 0.03 and 0.1 mg/kg produced a marked prolongation of duration time. Mer-A2026A (0.1 mg/kg), as well as papaverine (1 mg/kg), showed the similar potent depressor effects, but the duration time caused by Mer-A2026A was significantly longer than that by papaverine.

Discussion

In order to develop a new agent with vasodilating and depressor effects, we have found Mer-A2026A and B from a cultured broth of *S. pactum*.

The vasodilating effects of Mer-A2026A and B were examined using the muscle preparations of thoracic aortae from rats. Mer-A2026A and B showed a potent vasodilating activities in the muscles precontracted with NE or H-K, and the relative potency of both agents were greater than that of papaverine. Further, we injected the Mer-A2026A to SHR to examine the vasodilating activity *in vivo*. Mer-A2026A showed a marked depressor effect in SHR. The prolongation of hypotention was observed more markedly in the rats treated with Mer-A2026A than those with papaverine. These results suggest that Mer-A2026A shows a potent depressor effect as a result of direct vasodilating action of vascular smooth muscles. This hypothesis is supported by an preliminary experiment showing that Mer-A2026A strongly relaxes canine peripheral arteries such as femoral and coronary arteries (unpublished data).

Acute toxicity of Mer-A2026A was examined with ICR male mice. The LD_{50} value of this agent in intravenous dosage was more than 0.5 mg/kg.

In summary, we have found the Mer-A2026A and B with potent vasodilating activities.

Acknowledgments

The authors are grateful to Dr. NORIO SHIBAMOTO, Dr. MICHIKO SAKAMOTO, Dr. KUNIO ISSHIKI and Dr. NAOKI AGATA for their helpful advice, and Mr. YUTAKA HARA for his excellent technical assistance.

References

1) TAYLOR, H. D., L. KNOCHE & W. C. GRANVILLE (*Ed.*): Color Harmony Manual, Container Corporation of America, Chicago, 1948
2) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some *Actinomycetales* as an aid for species determination. J. Bacteriol. 56: 107~114, 1948
3) LECHEVALIER, M. P. & H. A. LECHEVALIER: Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 20: 435~443, 1970
4) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
5) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Int. J. Syst. Bacteriol. 18: 69~189, 1968