STUDIES ON NEW VASODILATORS, WS-1228 A AND B

I. DISCOVERY, TAXONOMY, ISOLATION AND CHARACTERIZATION

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New vasodilators, designated WS-1228 A and B have been discovered in the culture filtrate of a strain of *Streptomyces aureofaciens*. The active compounds were purified by column chromatography with Diaion HP-20 and silica gel, and finally separated from each other by high performance liquid chromatography. They were obtained as pale yellow crystals and their molecular formulae were both $C_{11}H_{17}N_3O$.

In the course of our screening program, a strain of *Streptomyces*, initially designated strain No. 1228, was found to produce the highly potent new vasodilators, WS-1228 A and B.

In this report, we describe the taxonomy of the producing organism, the fermentation and isolation procedures as well as the chemical and biological properties of these substances.

Taxonomic Studies on Strain No. 1228

Strain No. 1228 was isolated from a soil sample collected in Tokyo, Japan.

The methods described by SHIRLING and GOTTLIEB[1] were employed. Morphological observations were made with light and electron microscopy on cultures grown at 30°C for 14 days on yeast - malt extract agar, inorganic salts - starch agar and oatmeal agar. The mature spores occurred in chains of 10 to 30 spores forming *retinaculiaperti* or *spirals*. The spores were cylindrical and 0.4~0.8 by 0.9~1.6 μ in size with smooth surfaces.

Cultural characteristics were observed on ten kinds of media described by SHIRLING and GOTTLIEB[1] and WAKSMAN[2]. The incubation was made at 30°C for 14 days. As shown in Table 1, colonies belonged to the gray color series when grown on oatmeal agar, yeast - malt extract agar and inorganic salts - starch agar. No soluble pigment was produced.

Whole cell hydrolysates of strain No. 1228 contained LL-diaminopimelic acid. Summarized physiological properties of strain No. 1228 are shown in Table 2. Starch hydrolysis, milk coagulation and melanine production were negative. Gelatin liquefaction and milk peptonization were weakly positive. Utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[3]. The results were obtained after 14 days incubation at 30°C. As shown in Table 3, almost all carbon sources were doubtfully or not utilized. Only glycerin and sodium citrate were utilized.

Microscopic studies and cell wall composition analyses of strain No. 1228 indicated that this strain belongs to the genus *Streptomyces*. Accordingly, a comparison of this organism was made with the published descriptions[4~6] of various *Streptomyces* species. Strain No. 1228 is considered to resemble *Streptomyces olivaceus, Streptomyces aureofaciens, Streptomyces xantholiticus* and *Streptomyces viridifaciens*. It was found however, that strain No. 1228 could be differentiated from these species as follows:

Streptomyces olivaceus: No characteristic color is formed in substrate mycelia. No soluble pig-

Table 1. Cultural characteristics of strain No. 1228.

Medium	Aerial mass color	Reverse side of colony	Soluble pigment
Sucrose - nitrate agar	Very thin, powdery	Colorless, small colonies	None
Glucose - asparagine agar	Very thin, powdery	Yellowish brown small colonies	None
Glycerin - asparagine agar	None	Dull yellow orange ~ light brown, small colonies	None or trace
Starch - inorganic salts agar	Light gray, powdery	Yellowish brown ~ light brown, small colonies	Yellowish orange
Tyrosine agar	None	Light brown ~ brown, small colonies, slightly wrinkled	None
Nutrient agar	None	Pale yellow ~ reddish yellow, flat	None
Yeast - malt extract agar	Gray or pale yellow, powdery	Yellowish brown ~ brown, small colonies	Faint dull orange
Oatmeal agar	Gray, thin powdery	Colorless, small colonies	None or trace of yellow
Glucose - peptone gelatin stab	None	Colorless ~ cream, weak growth	None
Milk	White, thin powdery	Pale yellow, growth on surface	Trace
Peptone - yeast iron agar	None	Pale yellow ~ colorless, wrinkled colonies	None

Table 2. Physiological properties of strain No. 1228.

Properties observed	Characteristics
Temperature requirement	13 ~ 37°C, optimum 26°C
Gelatin liquefaction	Extremely weak
Starch hydrolysis	No hydrolyzed
Action on milk	No coagulation, Weak peptonization
Melanin production	None

Table 3. Utilization of various carbon componds.

L-Arabinose	−	Sucrose	±
D-Fructose	±	Salicin	−
Cellulose	−	D-Xylose	±
D-Glucose	±	Chitin	−
D-Galactose	±	Glycerin	+
Inositol	±	Lactose	±
D-Mannose	±	Maltose	±
D-Mannitol	−	Inulin	−
L-Rhamnose	±		
Raffinose	−		

+: good utilization.
±: doubtful utilization.
−: no utilization.

ment is produced in the medium. Utilization of carbon sources is good.

Streptomyces aureofaciens: No soluble pigment is produced.

Streptomyces xantholiticus: Growth of aerial mycelia is not abundant. Form of aerial mycelia is different from that of strain No. 1228. The color of substrate mycelia is greenish.

Streptomyces viridifaciens: Substrate mycelia show no characteristic color and its physiological characteristics are different from those of strain No. 1228.

As a result of comparisons, the microbiological characteristics of strain No. 1228 are in good agreement with those of *Streptomyces aureofaciens*, except for soluble pigment production. This difference, however, does not seem to us sufficient to distinguish strain No. 1228 from *Streptomyces aureofaciens*. And the strain is identified as *Streptomyces aureofaciens*. However, it is a new strain in that it produces the new biologically active substances, WS-1228 A and B. This strain has been deposited in the American Type Culture Collection as ATCC No. 31442.

Table 4. Media used for production of WS-1228 A and B.

Seed medium		Production medium	
Potato starch	1 %	Soluble starch	3 %
Gluten meal	1	Gluten meal	0.5
Corn steep liquor	0.5	Peanut meal	0.5
Dried yeast	0.5	Dried yeast	0.5
pH adjusted to 7.0 with 6 N NaOH		Na_2CO_3	600 μg/ml

Fermentation

Seed flasks containing 100 ml of the seed medium were inoculated with spores from the slant culture of strain No. 1228 and incubated at 30°C on a rotary shaker with 7.6-cm throw at 220 rpm for 3 days. A 30-liter fermentor with 20 liters of production medium was inoculated with 2~5% of the mature seed broth. The compositions of the seed and the production media are shown in Table 4. The fermentation was carried out at 30°C for 3 days under aeration of 20 liters/minute and agitation of 250 rpm. The progress of the fermentation was monitored by bioassay of the vasodilating activity.

Isolation Procedures

The flow diagram of the isolation method described below is shown in Fig. 1. Since most of the activity was present in the broth filtrate, the culture broth was filtered using filter aid (Radio-lite). The pH of the filtrate (18 liters) was adjusted to 6.8 with 6 N HCl and the active principles were adsorbed on a Diaion HP-20 column (1.5 liters). After washing with 3 liters of water, the column was eluted with 3 liters of methanol. The active fractions were concentrated under reduced pressure to dryness and applied to a column of silica gel (250 ml). After washing with 1 liter of benzene, the active components were eluted from the column with 500 ml of a mixture of benzene - ethyl acetate (4: 1, v/v). The active fractions were concentrated under reduced pressure to a volume of 20 ml to give 150 mg of pale yellow crystals. Since the crystals were mixture of WS-1228 A and B, they were subjected to high performance liquid chromatography under the condition shown in Table 5. Chromatography yielded two fractions: fraction A (retention time: 10 minutes) and fraction B (retention time: 12.5 minutes).

Fig. 1. Extraction and purification.

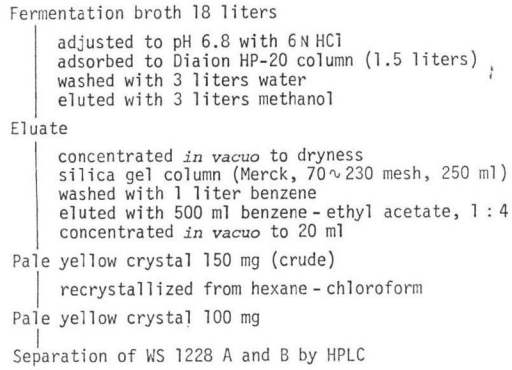

Table 5. High performance liquid chromatography of WS-1228 A and B.

Apparatus	Model M6000-A pump, U6K injector and 440 absorbance detector (Waters Associates Ltd.)
Stationary phase	Merk RP-18
Length of column	500 mm
Internal diameter of column	8 mm
Mobile phase	75% of Methanol
Column temperature	Ambient
Column pressure	141 kg/cm^2
Flow rate	5 ml/minute
Detector	Ultraviolet absorption at 254 nm

Table 6. Physicochemical properties of WS-1228 A and B.

	WS-1228 A	WS-1228 B
Appearance	Yellow needles	Yellow needles
Mp (°C)	100~102	135~138
Molecular formula	$C_{11}H_{17}N_3O$	$C_{11}H_{17}N_3O$
Molecular weight	207	207
Elemental analysis		
Found	C 63.88, H 8.28, N 20.09	C 63.46, H 8.23, N 20.51
Calcd.	C 63.74, H 8.27, N 20.27	C 63.74, H 8.27, N 20.27
UV λ_{max} nm (ε)	300 (44,000)	339 (43,000)
Color reaction	$FeCl_3$	$FeCl_3$

Table 7. Chromatographic behavior of WS-1228 A and B.

Solvent system	Rf
Benzene - ethyl acetate (3: 1, v/v)	0.45
n-Hexane - acetone (2: 1, v/v)	0.3

TLC: Silica gel.

Table 8. Relaxation of rat aorta superfused with Tyrode solution containing noradrenaline (0.03 μg/ml).

Sample	NA tone[1]	
	Relaxation (mg)	Duration (minute)
WS 1228 A 0.05 μg	No response	—
0.5	45	5
5	300	20
WS 1228 B 0.05 μg	175	10

1) The aorta was separated from male SD rat weighing 250 g and superfused with Tyrode solution containing noradrenaline (0.03 μg/ml) which increased the tension of the tissue by 500 mg.

From fraction A crystals of WS-1228 A (30 mg) and from fraction B were obtained crystals of WS-1228 B (3 mg).

Physicochemical Properties

The physicochemical properties of WS-1228 A and B are summarized in Table 6. Both compounds are soluble in methanol, ethanol, acetone, ethyl acetate, sparingly soluble in ether, benzene, *n*-hexane and insoluble in water. Rf values of WS-1228 A and B on TLC are presented in Table 7.

Color reactions are as follows: positive in sulfuric acid, iodine vapor, ferric chloride and potassium permanganate tests and negative in ninhydrin, EHRLICH and MOLISCH tests.

The chemical structures of these compounds will be described in the succeeding papers.[7,8]

Biological Properties

Male Sprague-Dawley rats of 8~10 weeks age were killed by a blow on the head and the thoracic aorta was quickly removed. After removing fatty tissues, spiral strips (2 mm width and 50 mm length) were made from the aorta and were suspended under a resting tension of 1 gram in 30 ml organ baths containing warm (37°C) oxygenated (95% O_2: 5% CO_2) Tyrode solution of the following composition: NaCl 137 mM (8 g/liter), KCl 2.7 mM (0.2 g/liter), $CaCl_2 \cdot 2H_2O$ 1.8 mM (0.264 g/liter), $MgCl_2 \cdot 6H_2O$ 1.02 mM (0.208 g/liter), $NaHCO_3$ 11.9 mM (1 g/liter), $NaH_2PO_4 \cdot 2H_2O$ 0.42 mM (0.066 g/liter) and glucose 5.55 mM (1 g/liter).

The tissues were equilibrated for almost 90 minutes and then were superfused with Tyrode solution containing a low concentration of noradrenaline (0.03 μg/ml) or KCl (30 mM) which increases the tension of the tissues by about 500 mg. Changes of tension of the tissues were measured isometrically by means of force displacement transducers coupled to a polygraph. Considerable variations in individual data were observed between different preparations, but fairly constant value was obtained for a preparation.

The relaxation activity of WS-1228 A and B is shown in Table 8. The activity of WS-1228 B is

Table 9. Relaxation of rat aorta superfused with Tyrode solution containing noradrenaline (0.03 μg/ml) or KCl (30 mM).

Sample (μg/ml)	NA tone[1]		KCl tone[2]	
	Relaxation (mg)	Duration (minute)	Relaxation (mg)	Duration (minute)
WS 1228 B				
0.1	420	10	200	20
0.05	190	10	N. T.	
Nifedipine				
0.15	150	45	N. T.	
0.075	N. T.		300	50

N. T.: Not tested.

1) The aorta was separated from male SD rat weighing 270 g and superfused with Tyrode solution containing noradrenaline (0.03 μg/ml) which increased the tension of the tissue by 490 mg.

2) The aorta was separated from male SD rat weighing 260 g and superfused with Tyrode solution containing KCl (30 mM) which increased the tension of the tissue by 510 mg.

markedly greater than that of WS-1228 A. The comparative activity of WS-1228 B with the calcium antagonist, nifedipine is summarized in Table 9. Calcium antagonists such as nifedipine are reported to show stronger relaxation activity on KCl treated than noradrenaline treated aorta. In repeated experiments the tendency was observed that WS-1228 B showed the reverse order of activities though not remarkable on the two preparations. The relaxation mechanism of WS-1228 B would appear to be different from that of calcium antagonists.

WS-1228 B shows no antimicrobial activity at a concentration of 100 μg/ml by the agar dilution method against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Candida albicans*.

Acute toxicity of WS-1228 B in *dd*Y mice is above 250 mg/kg by intraperitoneal injection.

Discussion

Microorganisms produce many useful antimicrobial and antitumor antibiotics[9]. Recently, many enzyme inhibitors were isolated from microbial products[10] and they are reported to have several unique activities in experimental animal models. Some of them are being applied to clinical trials.

In the screening mentioned in this report, we used the superfusion technique[11,12] which is frequently used in research on prostaglandins[13]. By using this method, we were able to detect active compounds quantitatively in a short time. The discovery of WS-1228 A and B produced by a strain of *Streptomyces* points out that the search in microbial products for new and novel prototype compounds for potential development as new drugs is promising when convenient and reliable methods can be employed. The vasodilating activity of WS-1228 B is now under investigation in animal models of hypertension.

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