STUDIES ON ANTIMICROBIAL SUBSTANCE B 44 P (STREPTOVARICIN) PRODUCED BY A STRAIN OF ACTINOMYCETES. I

PRODUCTION, EXTRACTION AND CHARACTERISTICS OF SUBSTANCE B 44 P AND THE IDENTITY OF THE SUBSTANCE WITH STREPTOVARCIN

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A pigment antibiotic, substance B 44 P, was obtained from the cultural liquid of *Streptomyces* species No. B 44-P 1 almost identified with *Streptomyces spectabilis*. Seven components were detected in the substance B 44 P by thin layer chromatography. Two main components (A, B) and a minor one (C) being identical with streptovaricins were purified.

During the course of screening for antibiotics, a low toxic antibiotic active mainly against Gram positive bacteria including mycobacteria was obtained from the cultured broth of a strain of soil actinomycetes (Strain No. B 44-P 1). This antibiotic named substance B 44 P was identified with streptovaricin[1,2,3] by the further study, but its low toxicity aroused the authors' interest in the further biological and pharmacological investigations and experimental chemotherapy.

In this report, the characteristics of the strain of *Streptomyces* No. B 44-P 1 and production, isolation and properties of the substance B 44 P are presented.

Mycological Properties of *Streptomyces* Species No. B 44-P 1

The strain No. B 44-P 1 was isolated from a soil sample collected in Genova, Italy. Its cultural characteristics were cited as follows:

Microscopical characters: Long straight filamentous aerial mycelium with a few branches; no spiral and no whorl; spore ellipsoidal, surface smooth.

Characters on various media: CZAPEK sucrose agar (27°C); slightly pinkish growth; pinkish, cottony aerial mycelium; no soluble pigment.

CZAPEK cellulose agar (27°C): Orange, spreading growth; aerial mycelium pale pink; no soluble pigment.

Glucose asparagine agar (27°C): Dark orange, wrinkle growth; no aerial mycelium; no soluble pigment.

BONNETT's agar (27°C): Dark orange, wrinkle growth; yellowish brown, powdery aerial mycelium; slightly yellow soluble pigment.

Calcium malate agar (27°C): Same as on glucose asparagine agar.

Nutrient starch agar (27°C): Dark orange growth with wrinkle surface; no aerial mycelium; no soluble pigment; hydrolysis positive.

Nutrient nitrate broth (27°C): Growth at bottom; brown soluble pigment; no nitrate reduction.

Skim milk (37°C): Surface ring orange growth; no coagulation or peptonization.

Gelatin stab (24°C): Purplish brown soluble pigment; no liquefaction.

Utilization of carbon sources: Glycerol, glucose, mannose, galactose, starch, trehalose were well utilized. Mannitol, maltose, sucrose, lactose, raffinose, dextrin were utilized. Arabinose, xylose, rhamnose, sorbitol, inulin were not utilized. The procedure followed that of PRIDHAM and GOTTLIEB[4].

The strain No. B 44-P 1 had no spiral and no whorl. Orange growth was obtained on synthetic agar. Pigment granules were produced in both substrate and aerial mycelium.

Comparison of the characteristics of strain No. B 44-P 1 with those of known species of *Streptomyces* indicated that strain No. B 44-P 1 was almost identical with *Streptomyces spectabilis*[2,5]. However, it was different from *Streptomyces spectabilis* in points of no liquefaction of gelatin, no coagulation of milk, no utilization of xylose and production of a toxic antibiotic[6] instead of actinospectacin[7].

Production of Substance B 44 P

The potency of the substance B 44 P produced was assayed by cylinder-plate method using *Staphylococcus aureus* 209 P as a test organism.

Examples of the experiments testing the influence of nitrogen and carbon sources on the production of the antibiotic in shaking culture at 27°C are indicated in Tables 1 and 2. From these results, a medium containing starch 2 %, glucose 2 %, dry yeast 1.5 %, NaCl 0.3 %, K_2HPO_4 0.1 % and $MgSO_4 \cdot 7H_2O$ 0.05 % was used for the

Table 1. The influence of nitrogen sources on the production of substance B 44 P

Nitrogen sources	%	2 nd day		3 rd day		4 th day		5 th day		6 th day	
		pH	mcg/ml	pH	mcg/ml	pH	mcg/ml	pH	mcg/ml	%	mcg/ml
Soybean	1.5	6.0	18.0	6.6	20.5	7.0	21.5	7.8	19.5	8.0	17.5
Peptone	1.5	5.8	17.8	7.8	21.8	8.6	20.8	8.6	13.5	8.8	10.5
Meat extract	1.5	5.6	17.5	6.4	20.4	7.4	21.3	8.6	17.0	8.8	14.0
Corn steep liquor	1.5	6.2	19.0	7.4	19.9	8.0	21.0	8.2	13.8	8.5	10.5
Yeast extract	1.5	6.4	18.3	7.4	23.5	7.6	25.5	8.2	18.8	8.4	16.0
$NaNO_3$	0.5	6.2	13.5	7.2	15.0	8.0	13.0	8.6	0	8.8	0
$(NH_4)_2SO_4$	0.5	4.4	10.0	4.2	11.0	4.4	0	4.8	0	5.3	0
$(NH_4)_2HPO_4$	0.5	4.6	13.5	5.0	14.0	5.4	12.0	6.0	10.0	6.2	0

Basal medium: Starch 1.0 %, NaCl 0.3 %, K_2HPO_4 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.05 %

Table 2. The influence of carbon sources of the production of substance B 44 P

Carbon sources	%	2 nd day		3 rd day		4 th day		5 th day		6 th day	
		pH	mcg/ml	pH	mcg/ml	pH	mcg/ml	pH	mcg/ml	pH	mcg/ml
Glucose	2	5.0	3.1	6.0	11.0	7.6	22.6	7.8	18.7	8.0	19.6
Lactose	2	8.0	13.5	8.4	10.0	8.6	0	8.8	0	8.8	0
Sucrose	2	8.4	7.2	8.5	0	8.8	0	8.9	0	8.9	0
Maltose	2	8.0	12.7	8.0	10.1	8.2	0	8.4	0	8.6	0
Dextrin	2	6.0	7.4	6.8	12.8	7.6	4.5	8.0	0	8.2	0
Starch	2	6.2	6.5	6.4	14.8	7.0	38.0	7.6	42.2	8.0	26.4
Glycerin	2	6.2	3.1	6.2	11.0	6.2	12.5	6.6	16.2	7.6	22.2
Glucose+Starch	1+1	5.4	20.5	6.2	54.0	7.0	80.0	7.6	81.0	8.0	52.8
Glucose+Starch	2+1	5.4	6.5	5.0	21.0	5.8	26.2	5.8	77.7	7.2	67.2
Glucose+Starch	1+2	5.4	16.5	5.8	33.0	6.0	58.5	6.2	96.0	7.4	77.8
Glucose+Starch	2+2	5.2	21.8	5.6	48.4	5.6	71.0	5.8	103.5	5.8	91.5

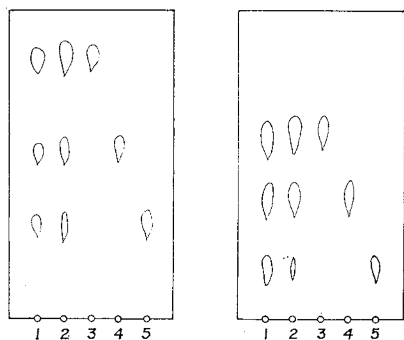
Basal medium: Yeast extract 1.5 %, NaCl 0.3 %, K_2HPO_4 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.05 %

fermentation. The medium of 170 liters was placed in a fermentor of 400-liter capacity and sterilized at 120°C for 25 minutes. Soybean oil 0.1 % and silicon oil KM-66 (Shinetu Chemical Co., Ltd.) 0.005 % were added as anti-foamers. After cooling, the medium was inoculated with 1 liter of 48-hour shaking seed and incubated at 28°C for 3 days with continuous stirring and aeration. The antimicrobial activity of the fermented broth indicated 61.5 mcg/ml.

Isolation and Purification of Substance B 44 P

After 70-hour fermentation, the whole beer was filtrated at harvest pH 6.6 and the filtrate (160 liters) was extracted with 100 liters ethyl acetate at adjusted pH 4.2. Acetate extract (90 liters) was distilled under reduced pressure to a volume of 900 ml. The concentrate was filtered and the filtrate was treated with 4,500 ml of n-hexane to give a precipitate. The precipitate collected by filtration was dissolved in chloroform and insoluble part was removed by filtration after standing overnight at 5°C. The filtrate was treated again with four volumes of n-haxane and the orange precipitate obtained was dried *in vacuo*. Crude substance B 44 P possessing a potency of 323 mcg/mg was obtained in an amount of 16.25 g and the yield in the powder was about 52 %. As shown in Fig. 1, on a paper chromatography of the powder in a solvent system of cyclohexane - chloroform - water (1 : 8 : 2) using filter paper previously treated with a citric acid-phosphate buffer (pH 4.1) and in a solvent system of benzene - methanol - water (1 : 1 : 2), it was noted that three components (A, B, C) were presented in the crude powder by bioautography against *Staphylococcus aureus*.

Fig. 1. Paper chromatogram of Substance B 44 P.



Solvent system : cyclohexane-chloroform-water (1 : 8 : 2)

Solvent system : benzene-methanol-water (1 : 1 : 2)

1. Streptovaricin complex
2. Substance B 44 P complex
3. Substance B 44 P component A
4. Substance B 44 P component B
5. Substance B 44 P component C

Five grams of the crude complex dissolved in 80 % aqueous acetone was passed through a column of activated carbon and the column was washed with the same solvent until the antibiotic was completely eluted. Fractions of the effluent which exhibited the antibacterial activity were collected and then dried *in vacuo*. The residue was dissolved in chloroform and the impurity was removed by adsorption on silica gel. After filtration, the filtrate was concentrated to dryness *in vacuo*. There was obtained 1.5 g of the crude powder possessing an activity 75 % as much as that of pure component A and yield in this procedure was about 70 %. The crude powder thus obtained gave two main active spots (A, B) and minor ones (C, D, E, F, G) by thin-layer chromatography on silica gel using a solvent system of chloroform - methanol (100 : 5). In this case, the content ratio of the components A, B and C in the complex was 65 : 26 : 1, when measured by the inhibitory activity against *S. aureus*, and other components were observed in trace amounts.

In order to separate these main components, 5 g of the crude powder treated by carbon and silica gel was chromatographed on silica gel using mixed solvent of chloroform and methanol (98 : 2). The component A was eluted first, followed by B. After each fraction of the effluent was confirmed to give one spot on thin layer chromatography, solvent was removed to dryness. The residue was twice recrystallized from ether. Thus 1.33 g of substance B 44 P A and 0.23 g of B were obtained. The component C was separated as considerably pure amorphous material in a small amount by repeated thin-layer chromatography and subsequent precipitation from ether. The separation of the other components was unsuccessful.

Properties of Substance B 44 P

Substance B 44 P is a complex of closely related components, each of which possesses antibiotic activity. Three separated components A, B and C and four minor components are found in the complex. The determination of these components by paper chromatography and thin-layer chromatography was described above.

Substance B 44 P A is reddish orange crystalline powder. It melts at 186~191°C. Specific rotation $[\alpha]_D^{20}=+590°$ (chloroform). As shown in Fig. 2, the ultraviolet absorption spectrum in methanol solution has maxima at 245 mμ ($E_{1cm}^{1\%}$ 630) and 430 mμ ($E_{1cm}^{1\%}$ 190). Infrared spectrum in KBr is shown in Fig. 3. It is soluble in methanol, ethanol, acetone, chloroform and ethyl acetate, slightly soluble in ether, benzene and water and insoluble in *n*-hexane. It gives positive ferric chloride reaction and negative BENEDICT, ninhydrin, MOLISCH, SAKAGUCHI and FEHLING reaction. Its aqueous solution is yellow in acid but turns to orange in alkali. It is fairly stable at a pH range of 2.0 to 6.0 and about 90 % of the activity are retained at pH 4.0 after standing at 60°C for 2 hours. It is unstable in alkaline solutions. There is complete loss in activity at pH 10 after heating at 60°C for 30 minutes.

Fig. 2. Ultraviolet absorption spectrum of substance B 44 P component A in methanol.

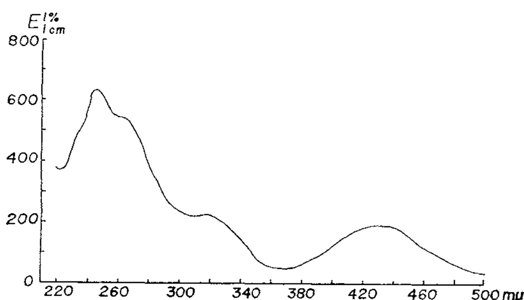
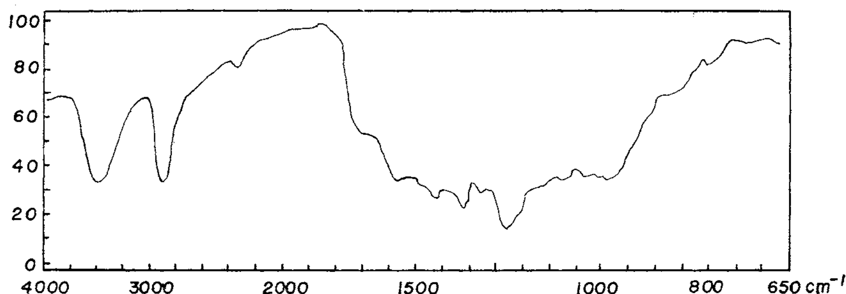


Fig. 3. Infrared absorption spectrum of substance B 44 P component A (KBr tablet).



Substance B 44 P B melts at 182~185°C. Specific rotation $[\alpha]_D^{20}+450°$ (chloroform). Substance B 44 P C melts at 185~191°C. They were closely related to component A in the chemical, physical and biological properties.

An acute intraperitoneal toxicity test in mice using a complex gave an LD_{50} value of 375 mg/kg. Mice tolerated to subcutaneous injection of 1,000 mg/kg and oral dose of 2,000 mg/kg.

The details on antimicrobial activity are shown in another report[8].

When the substance B 44 P was compared with known pigment antibiotics, the characteristics of the substance B 44 P A, B and C were considered to be identical with those of streptovaricin C, B and A respectively.

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