STUDIES ON ANTIBIOTICS PRODUCED FROM *KITASATOA KAUAIENSIS* SP. NOV.

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Kitasatoa kauaiensis, a new species of the genus *Kitasatoa*, belonging to the family *Streptoplanaceae*, produces 3 kinds of antibiotics in a culture broth. These antibiotics were identified as chloramphenicol, bottromycin and fradicin based on their physico-chemical and biological properties. A new differential bioassay method for chloramphenicol and bottromycin produced in the same culture broth was established.

A strain of microorganism No. I-337, which was isolated from a soil sample in Kauai Isle of Hawaii Islands, differs from the genus *Streptomyces* in morphological findings. This new species belonging to a new genus *Kitasatoa* in the family *Actinoplanaceae* was named *Kitasatoa kauaiensis*, as reported in the separate paper[1]. This strain was found to produce three kinds of antibiotics in the culture broth by a paper chromatography. Two antibiotics, I-337 A and B possess antibacterial activities against *B. subtilis* PCI 219, and the other antibiotic, I-337 C possesses antifungal activity against *Saccharomyces cerevisiae*.

The authors extracted three effective substances from a culture filtrate of the strain. On investigating physico-chemical and biological properties of these antibiotics, I-337 A was identified as chloramphenicol, B as bottromycin[2], and C as fradicin[3,4] respectively.

When No. I-337 strain is inoculated, I-337 A is often produced with B in the same culture broth. It became necessary to estimate both antibiotics I-337 A and B separately in the same culture filtrate for the purpose of increasing the amount of I-337 B. I-337 A showed a high activity to both Gram-positive and Gram-negative bacteria, while I-337 B showed an activity only to the Gram-positive bacteria and indicated a clear inhibition zone of *B. subtilis* at pH 8. A new differential bioassay method was established using *E. coli* as test organism for I-337 A and *B. subtilis* for I-337 B. Present paper deals with extraction and isolation of I-337 A, B and C and with a differential assay method of chloramphenicol and bottromycin in the same culture broth.

Materials and Methods

(1) Fermentation

The antibiotic-producing strain, No. I-337, was maintained on WAKSMAN's or KRAINSKY's agar slants. The inoculum for a jar fermentor was incubated for 48 hours at 27°C on a reciprocal shaker.

A seed (0.5 %) was inoculated into 20 liters of culture medium in a 30-liter stainless-steel jar fermentor and the fermentation was carried out at the following conditions: temperature, 28°C; aeration 20 liters/min.; agitation, 230 rpm; and pressure, 0.5 kg/cm^2.

Samples were obtained at 12-hour intervals to observe pH, mycelial volume, carbohydrate utilization and antibacterial activity.

(2) Estimation of Antibacterial Activities of Antibiotics.

The antibacterial activity of I-337 A and B was estimated by a paper disc plate metod using *E. coli* grown on nutrient agar at pH 7 and *B. subtilis* PCI 219 grown on nutrient agar at pH 8, respectively.

Agar plates for the paper disc method were prepared as follows: Ten ml of nutrient agar medium for base layer and 4 ml of seed agar medium for seed layer were dispensed into each Petri dish.

(3) Paper Chromatography.

Paper chromatography of the broth filtrate was carried out on Tōyōroshi No. 50 paper strips (1×40 cm) by the ascending methods with 3 % ammonium chloride solution. The dried strips were bioautographed against *B. subtilis* PCI 219 on agar medium (pH 8). The Rf values of I-337 A and B were approximately 0.78 and 0.56 respectively.

Experimental Results

Isolation and Purification of I-337 A

A 20-liter batch of the broth was filtered and the filtrate was extracted successively with 6-liter and 4-liter portions of ethyl acetate. The extract was concentrated to dryness *in vacuo* and crude substance (2.5 g) obtained was dissolved in a minimal volume of acetone and purified by chromatography on a colum (2.8×15 cm) containing 100 g of aluminum oxide. The active fraction was eluted with 30 % methanol in chloroform and dried *in vacuo*. I-337 A (750 mg) was obtained as colorless needle crystals with acetone-chloroform and overall yield was 24 %.

Isolation and Purification of I-337 B and C

I-337 B and C were extracted from the fermentation broth in a 400-liter stainless-steel fermentor. The fermentation broth, fermented in the soybean meal medium for 3 days was filtrated and the filtrate (360 liters) was extracted two times with 80-liter and 40-liter portions of ethyl acetate at pH 7.2. The extract (80 liters) was concentrated to 17 liters and extracted four times with 0.1 M acetic acid solution (3.4 liters). Acetic acid phase (14.5 liters) was neutralized with 3 N sodium hydroxide and again extracted three times with ethyl acetate (3 liters) at pH 7.8. The extract was dried *in vacuo* to obtain a crude powder (5.9 g) containing I-337 B and C. The product thus obtained was purified by alumina column chromatography (120 g of aluminum oxide, column 2.5×15 cm) with chloroform as a developer. The active fraction (1.8 g) containing I-337 B and C was dried *in vacuo*. The active fraction was further purified by Sephadex LH20 column chromatography using methanol as a developer. I-337 B was eluted first, subsequently I-337 C. Each fraction was combined and concentrated *in vacuo*. I-337 B (1.5 g) was obtained as a white amorphous powder with overall yield 60 %. I-337 C (30 mg) was obtained as pale yellow needles from chloroform-methanol.

Identification of the Antibiotics

I-337 A was obtained as colorless needles with a melting point of 148~149°C by

Fig. 1. Two-dimensional cellulose thin-layer chromatogram of I-337B hydrolysate (6 N HCl, for 24 hours). Detected by ninhydrin.

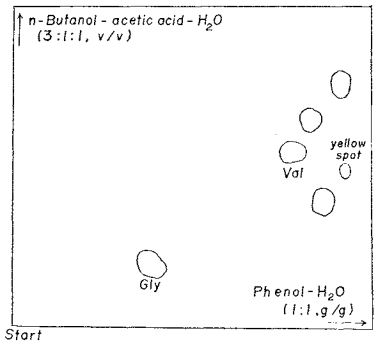
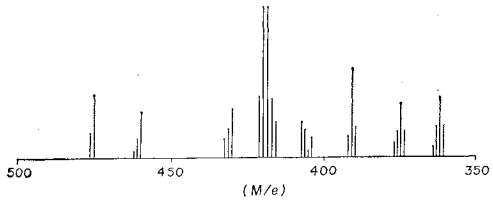


Fig. 2. Mass spectrum of I-337B.

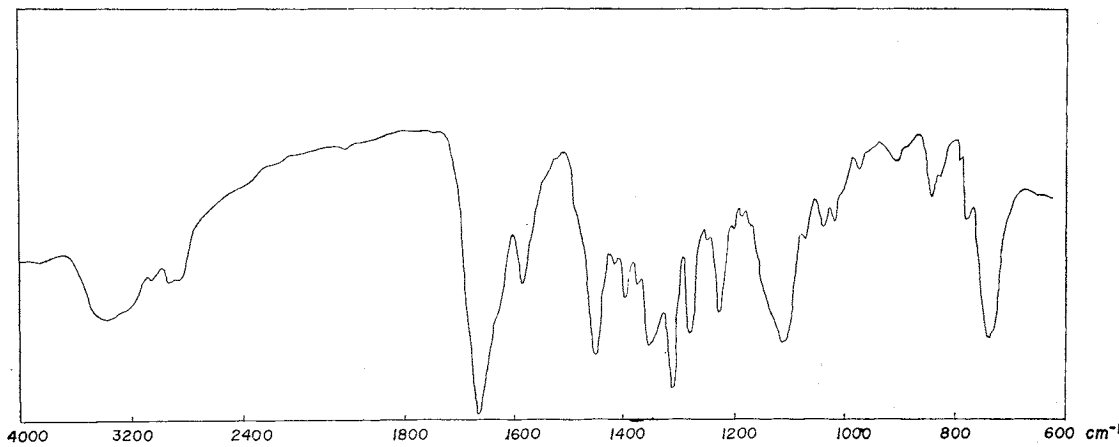


recrystallization. The ultraviolet absorption spectrum in methanol exhibits a maximum at 278 mμ ($E^{1\%}_{1cm}$ 350). The following elementary analysis was obtained: C 40.95, H 3.67, N 8.61. The antibiotic I-337 A was identified as chloramphenicol by the comparison of its infrared absorption spectrum and thin-layer chromatogram with those of the authentic sample of chloramphenicol.

I-337 B was a white amorphous powder with weak basic properties. The elementary analysis was as follows: C 60.89, H 7.58, N 12.59, S 4.18. The antibiotic had a specific rotation $[\alpha]_D^{25}$ $-32.1°$ (c 2, methanol). The potentiometric titration of I-337 B in methanol-water showed one titrable group, pK'a 7.01. The equivalent weight based on the titration was 715. The ultraviolet absorption spectrum of I-337 B in methanol showed an end absorption at 210 mμ. The presence of ester peptide bonds was indicated in the infrared absorption spectrum ($\nu_{C=O}$: 1745 cm^{-1}, KBr). The nuclear magnetic resonance spectrum showed a peak at δ=3.7 ppm, corresponding to O-methyl group.

By hydrolysis of I-337 B with 6 N hydrochloric acid at 110°C for 24 hours, six ninhydrin-positive spots were detected in the hydrolysate by two-dimentional cellulose thin-layer chromatography with solvent systems consisting phenol-water (3 : 1) and *n*-butanol-acetic acid-water (3 : 1 : 1). The chromatogram thus obtained is indicated in Fig. 1. Two spots were confirmed to be glycine and valine by com-

Fig. 3, Infrared spectrum of I-337C (KBr).



parison with their authentic samples, but others were not identified.

Based on the above facts, I-337 B was supposed to be identical with bottromycin.

When thin-layer chromatography using silica gel G was carried out with the solvent system of *n*-butanol - acetic acid - water (100 : 12 : 100) (upper phase) and the developed plates were exposed to iodine vapour, two yellow bands were observed. The Rf value of the main band was approximately 0.7, as same as that of an authentic sample of bottromycin A_2. The Rf value of the weak band was 0.65. This seems to be bottromycin B. The main band was eluted with methanol and obtained as a white amorphous powder. The mass spectrum of this fraction showed a peak at M/e 475 (Fig. 2). This peak accords with the molecular weight of Δ^1-isocaproyltetrapeptide obtained from bottromycin A_2 as reported by NAKAMURA *et al.*[5,6] From these data, I-337 B was identified as bottromycin A_2 containing a trace of bottromycin B.

I-337 C was pale yellow needles and did not show a definite melting point. It was darkened slowly at above 200°C. Analytical value was as follows: C 71.46, H 5.59, N 11.67. I-337 C showed a characteristic ultraviolet absorption in methanol at 245 ($E^{1\%}_{1cm}$ 660), 270 (sh), 295 (1720), 325 (sh), 337 (310), 356 (170), and 375 (250) mμ. The infrared absorption spectrum of I-337 C is shown in Fig. 3. I-337 C was identified as fradicin[4] from the properties described above.

Differential Assay between I-337 A and B

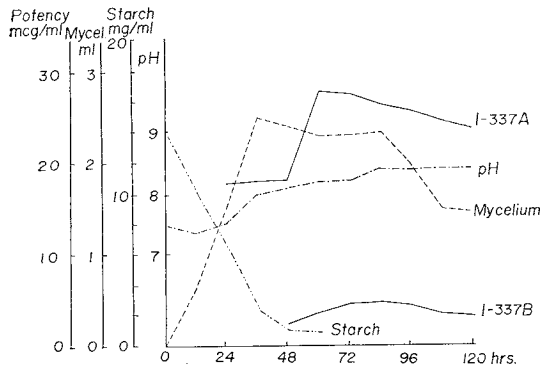
Based on the isolation method described above, a differential assay between I-337 A (chloramphenicol) and B (bottromycin) was performed as follows: Ten ml of aqueous solution of purified sample of I-337 A or B was extracted with an equal volume of ethyl acetate and the ethyl acetate solution was reextracted with 10 ml of 0.1 M acetic acid. The acetic acid phase was neutralized with 3 N sodium hydroxide. It was found by paper chromatography that I-337 A remained in the ethyl acetate

Table 1. Recovery tests

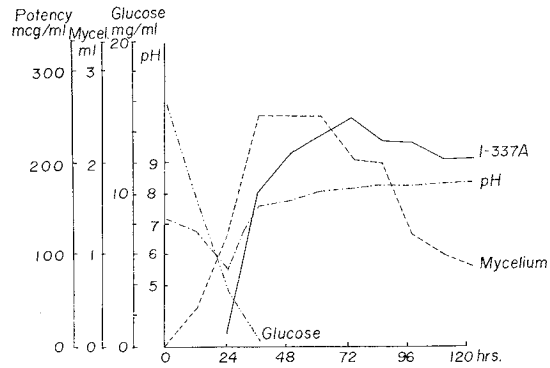
Test sample		Acetic acid or ethyl acetate (ml)	Potency (μg/ml)	Total potency (μg)	Total potency minus broth potency (μg)	Recovery ratio (%)
Broth filtrate	10 ml	11.4	10.8	123		
Broth filtrate Bottromycin	10 ml 200 μg	11.2	26	296	173	87
//	100 μg	11.4	18.2	207	84	84
//	50 μg	11.4	14.8	169	46	92
Distilled water Bottromycin	10 ml 200 μg	11.0	16	176		88
//	100 μg	11.4	7.6	87		87
//	50 μg	11.4	3.7	42		84
Broth filtrate	10 ml	8.5	16.5	140		
Broth filtrate Chloramphenicol	10 ml 500 μg	8.4	73.5	615	475	95
//	200 μg	8.6	38	327	187	94
Distilled water Chloramphenicol	10 ml 500 μg	8.4	60.7	510		102
//	200 μg	8.4	29.5	185		92

Fig. 4. Fermentation patterns.

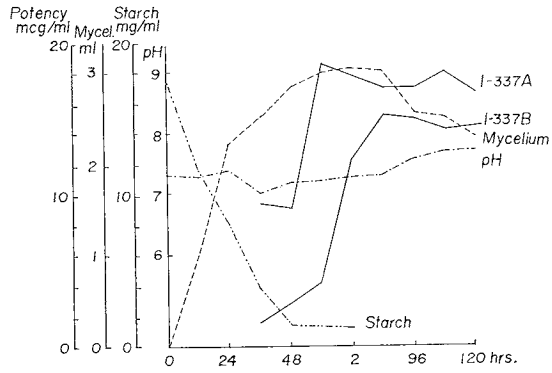
(1) Cornsteep liquor medium.
Cornsteep liquor 2 %, Starch 2 %,
Yeast 0.3 %, NaCl 0.5 %, $CaCO_3$ 0.3 %



(2) Peptone medium.
Peptone 2 %, Glucose 2 %, Meat extract
0.5 %, NaCl 0.5 %, $CaCO_3$ 0.3 %



(3) Soybean meal medium.
Soybean meal 2 %, Starch 2 %,
Yeast 0.3 %, NaCl 0.5 %, $CaCO_3$ 0.3 %



phase and I-337 B was transferred into dilute acetic acid. The antibacterial activity of the ethyl acetate phase (I-337 A) was estimated by a paper disc method against *E. coli* grown on nutrient agar at pH 7. The dose-response standard curve was prepared over the range of 6.25~100 μg per ml.

I-337 B was transferred into 0.1 M acetic acid phase and the activity was estimated using *B. subtilis* PCI 219 grown on nutrient agar at pH 8 as a test organism. The diameters of the clear inhibion zones were measured to prepare the standard curve. The standard curve was linear over the range of 1.6~100 μg per ml.

The fermentation broths could be assayed in similar fashion.

Recovery tests of I-337 A and B in the culture filtrate were performed by adding 500, or 200 μg of I-337 A, and 200, 100 or 50 μg of I-337 B to 100-ml portion of the broth filtrate or distilled water and assayed. The results are summarized in Table 1. The recovery of I-337 A was more than 90 % and that of I-337 B 84~92 %.

A factor of 1.13 was used to correct the values of I-337 B for the recovery found.

Fermentation Studies

The time course of the fermentation of I-337 A and B is summarized in Fig. 4.

With a medium containing soybean meal, the highest production of I-337 A and B was obtained after 60~84 hours of fermentation, and the potencies of I-337 A and B were on an average of 15~30 μg and 10~20 μg per ml, respectively.

With cornsteep liquor as a nitrogen source instead of soybean meal, the maximal yields of I-337 A and B were 28.0 and 5.2 μg per ml after 60~72 hours, respectively.

When the fermentation was performed with a medium containing peptone, I-337

A was the main product and no I-337 B was produced. A peak potency of 250 μg per ml was obtained at 72 hours.

The data seem to indicate that the production of I-337 A and B may be much more dependent on the nitrogen sources, than on the carbohydrates in the medium. The peptone medium was favorable for the production of I-337 A and the soybean meal medium for the production of I-337 B.

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