ANTIBIOTIC YC 73 OF PSEUDOMONAS ORIGIN. I

PRODUCTION, ISOLATION AND PROPERTIES

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A dark green antibiotic designated as YC 73 was isolated from the culture broth of a pseudomonad. The antibiotic ($C_4H_8N_2O_2S_2Cu$) proved to be hitherto unknown and is characterized by the presence of sulfur and copper atoms in its molecule. It is active against bacteria and fungi.

In the course of a systematic screening program for new antibiotics, an aerobic, fluorescent pseudomonad, indexed as MCRL 10107, was found to produce an antibiotic active against bacteria and fungi. The antibiotic, designated as YC 73 in our labotatory, has been isolated in the form of dark green crystals from culture broth of the producing strain. The compound turned out to be unknown up to now and is characteristic in the respect that it contains sulfur and copper.

The present paper deals with the production, isolation, purification and properties of the antibiotic YC 73. Taxonomic studies of the YC 73 producing strain, the structure and synthesis of YC 73 and of its analogous compounds will be reported later.

Production, Isolation and Purifiction

The antibacterial activity obtained during the production, isolation and purification processes, was assayed by an agar-cup-plate or paper-disc plate method with *Staphylococcus aureus* Terashima as a test organism. The purest sample of YC 73 was used as a standard material.

In a 2,000-liter fermentor, 1,200 liters of a medium composed of 1.2 % sucrose (granular sugar), 3.0 % soy bean meal, 0.2 % $(NH_4)_2SO_4$, 0.2 % $CaCO_3$ and 0.00075 % $CuSO_4 \cdot 7H_2O$ was adjusted to pH 7.0. After autoclaving followed by cooling, the medium was inoculated with a suspension of *Pseudomonas* MCRL 10107 prepared by suspending the cells of a nutrient agar slant culture in 100 ml of sterile physiological saline solution. The cultivation of the bacteria was carried out under the following conditions: temperature 26±1°C, inner pressure 0.5 kg/cm², aeration 600 liters/min., agitation 200 rpm. PBC-41 (Nikko Chemicals Co.) dissolved in liquid paraffin was used as an antifoam agent. The maximum accumulation of YC 73 was observed after 42 ~48 hours, when the pH of the broth was about 8.5 and the potency has reached a level of 60 mcg/ml of culture broth.

The isolation of the antibiotic from the fermented broth (1,200 liters, 60 mcg/ml) was achieved as follows: The broth was adjusted to pH 2.0 with 6 N HCl, and 120 kg of $(NH_4)_2SO_4$ was dissolved in it. Finally, the broth was filtered with the aid of 20 kg of diatomaceous earth. The filtrate was re-adjusted to pH 2.0, and extracted with 400 liters of ethyl acetate. The extract was concentrated *in vacuo* to give a syrup which was solidified with petroleum ether. The solid material was collected by filtration and dried *in vacuo*. Thus, 150 g of a crude powder of YC 73 (210 mcg/mg) were obtained with an over-all yield of about 45 %. Thin-layer chromatography with Kieselgel GF_{254} (detection of the antibiotic by visible color and bioautography) indicated that the powder contained a single active component, Rf values being 0.19 with chloroform and 0.57 with ethyl acetate.

Purification of the crude material was achieved by column chromatography. The sample (150 g) was dissolved in 200 ml of chloroform, poured onto a column packed with 2.0 kg of Kieselgel G previously moistened with water and dried in the air overnight and developed with chloroform. The eluates were monitored by thin-layer chromatography and bioassay. Antibiotically active colored fractions of the eluates were collected and concentrated *in vacuo* to a syrup, which turned to crystals by treating with ethyl ether. Crystals thus obtained were recrystallized from ethanol. Thus, starting from 150 g of a crude powder, 12 g of YC 73 (1,000 mcg/mg) were obtained in the form of dark green needles.

Properties

A crystalline sample of YC 73 melted at 199°C with decomposition. It was soluble in acetone, chloroform, dioxane, pyridine, dimethylformamide and dimethylsulfoxide, and slightly soluble in methanol, ethanol, ethyl acetate and water, and hardly soluble or insoluble in hydrocarbons and ether.

The molecular formula of YC 73 was confirmed to be $C_4H_8N_2O_2S_2Cu$ by elementary analysis and molecular weight determination.

Calcd : C 19.07, H 3.31, N 11.49, S 26.25, Cu 26.05. M.W. 243.8.
Found: C 20.02, H 3.25, N 11.71, S 25.31, Cu 27.23. M.W. 240 (VPO in chloroform)

Further data, especially on compounds related to YC 73 will be published later.

As shown in Fig. 1, the following absorption maxima of YC 73 were observed in its ultraviolet spectrum in methanol; λ_{max} ($E^{1\%}_{1cm}$): 231 (535), 253 (515), 267 (592), 320 (297) and 365 (shoulder, 129). There was no difference between absorption maxima measured in weakly acidic or weakly alkaline methanol. As shown in Fig. 2, the infrared absorption spectrum measured in a KBr disc showed the following frequencies: 3500, 2980, 1575, 1453, 1428, 1406, 1340, 1152, 1100, 910, 892 and 878 cm^{-1}.

Fig. 1. Ultraviolet spectrum of YC 73 in methanol

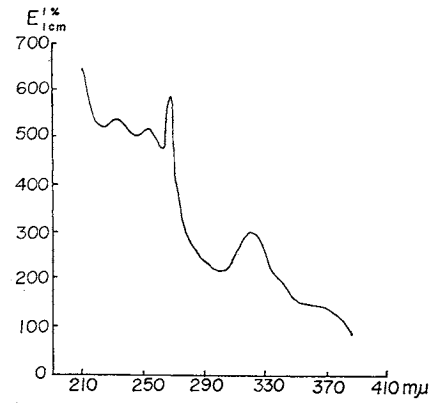
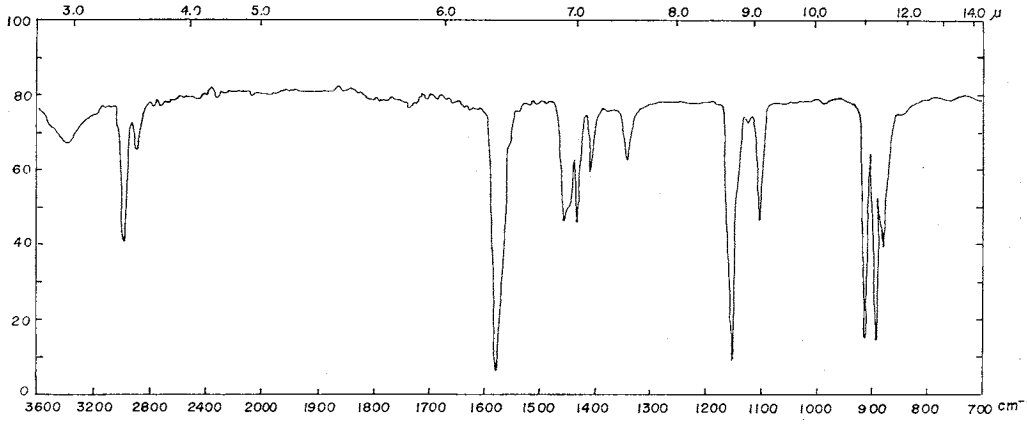


Fig. 2. Infrared spectrum of YC 73 (KBr)



Because of the dark green color of the antibiotic itself, color reactions for YC 73 were ambiguous. Color reactions gave the following results: TOLLENS, black precipitation; $FeCl_3$, blue green coloration in methanol and in MACILVEIN buffer solution (pH 7.0). Alkaline sodium nitroprusside solution: yellow red coloration in aqueous methanol, turning to blue green on addition of acetic acid. $KMnO_4$: red brown in acetone, decolored on addition of glacial acetic acid. MOLISCH: light green band at the boundary zone with sulfuric acid. FEHLING: brown precipitation on heating, supernatant turning green. Biuret: light blue, soon changing to light violet. Conc. sulfuric acid: no color change. Conc. hydrochloric acid: light yellow green. EHRLICH: light brown on heating. BENEDICT: green on heating. SAKAGUCHI: negative. Ninhydrin: negative.

Paper chromatograms (ascending method) on filter paper strips (Toyo Roshi No. 51A) gave Rf values as shown in Table 1. On paper electrophoresis (10 volt/cm, 2.5 hours in 1/15 M phosphate buffer), YC 73 moved slightly to the cathode at pH 5.0 and at pH 8.0.

Table 1. Rf-values of paper chromatograms (Bioautography with *Staphylococcus aureus* Terashima)

Solvent system	Rf value
Wet *n*-BuOH	0.73
20% Aq. ammonium chloride solution	0.65
50% Aq. acetone	0.85
n-BuOH (40 ml)-MeOH (10 ml)-H_2O (20 ml)	0.69
Benzene (40 ml)-MeOH (10 ml)	0.82
Distilled water	0.73

The antimicrobial activity of YC 73 is illustrated in the Table 2. As shown in the table, YC 73 is active against various kinds of Gram-positive and Gram-negative bacteria including pathogenic ones. It is also active against fungi. YC 73 was found to be considerably toxic to mice, LD_{50}: 3~6 mg/kg (ip), so that it would not be used as a chemotherapeutic agent, until any other useful derivatives or analogous compounds would be discovered.

Discussion

As described above, YC 73 is an antibiotic of a low molecular weight. It is further characterized by the presence of sulfur and copper as molecular constituents. More than thirty antibiotics including blue or green colored compounds produced by pseudomonas strains have been described in the literature[1,2]. However, no antibiotic was found which

contained sulfur. Furthermore, among antibiotics produced by other species of microorganisms, no antibiotic was found which proved to be identical with YC 73. Accordingly, YC 73 seems to be a new antibiotic.

As will be reported later, YC 73 is the cupric salt of a new low-molecular acidic antibiotic, C_2H_5NOS, which we propose to name thioformin. Therefore, YC 73 is properly called cupri-thioformin.

Table 2. Antimicrobial activity of YC 73 (Serial dilution method. MIC was determined after 24 hours, unless indicated otherwise)

Test Organisms	Medium [a]	MIC (mcg/ml)
Staphylococcus aureus FDA 209P	I	0.09
Staphylococcus aureus Terashima	I	0.09
Staphylococcus aureus Smith	I	0.09
Staphylococcus aureus R-1 [b]	I	0.09
Staphylococcus aureus R-2 [c]	I	0.09
Streptococcus hemolyticus	II	0.78
Diplococcus pneumoniae	III	0.78
Corynebacterium diphtheriae P. W. No. 8	II	1.56
Bordetella pertussis Tohama	III	0.19 (48 hours)
Neisseria meningitidis group A 13077	III	0.19
Neisseria meningitidis group B 13090	III	0.19
Neisseria gonorrhoeae	III	0.19
Bacillus subtilis PCI 219	I	0.09
Escherichia coli NIHJ	I	1.56
Klebsiella pneumoniae	I	3.12
Shigella dysenteriae	I	0.78
Shigella flexneri 2a	I	0.78
Salmonella typhosa T-58	I	0.39
Proteus vulgaris	I	0.19
Pseudomonas aeruginosa 1095	I	12.5
Mycobacterium tuberculosis $H_{37}Rv$	V	3.12 (3 weeks)
Clostridium welchii	IV	3.12
Clostridium tetani	IV	1.56
Willia anomala	VI	3.12
Hansenula anomala	VI	1.56
Torula utilis	VI	3.12
Saccharomyces cerevisiae	VI	0.78
Candida albicans	VI	3.12
Trichophyton mentagrophytes	VI	6.25
Trichophyton rubrum	VI	3.12
Trichophyton concentricum	VI	6.25
Epidermophyton floccosum	VI	3.12
Microsporum canis	VI	6.25
Aspergillus niger	VI	25.0
Penicillium notatum	VI	50.0

a) Medium used. I: nutrient broth (Difco), II: brain heart infusion broth (Difco), III: brain heart infusion broth enriched with 10% horse serum, IV: ZEISSLER's blood agar, V: KIRCHNER's medium enriched with 10% horse serum, VI: SABOURAUD's liquid medium.
b) Resistant to penicillin, chloramphenicol, tetracycline, erythromycin and sulfa drugs.
c) Resistant to penicillin, streptomycin and tetracycline.

References

1) KORZYBSKI, A.; Z. KOWSZYK-GINDIFER & W. KURYŁOWICZ: Antibiotics. Origin, nature and properties. Vol. 1, pp. 9~21, Pergamon Press, London, 1967
2) RAO, G. S.; B. B. BANNUR & G. M. PURANDARE: Index to antibiotics producing microorganisms. Hindustan Antibiot. Bull. 10: 7~122, 1967

Addendum

Experiments on the isolation and characterization of the antibiotic YC 73 were completed at the end of 1968. Independently to us, Dr. T. ROKUTANDA, Kumamoto University, Department of Medicine, and his collaborators (the 42nd Annual Meeting of the Japan Bacteriological Society, April 8, 1969) reported on a copper-containing antibiotic produced by a pseudomonad in a medium containing $10^{-2} \sim 10^{-3}$ molar copper ions (cf. Jap. J. Bacteriol. 24: 587, 1969).

Thanks to Dr. T. DOKE, one of the collaborators of Dr. T. ROKUTANDA, we were afforded with their antibiotic substance PS 66 together with its producing strain, *Pseudomonas* sp. No. 66. We were also supplied with other *Pseudomonas* strains which produced antibiotic substance(s) in a copper-containing medium. As a result of comparative experiments, not only PS 66, but also antibiotics produced by other *Pseudomonas* spp. were found to be identical with YC 73 described in the present paper.