STUDIES ON *ACTINOMYCES TUMEMACERANS* STRAIN INMI P-42 WITH PARTICULAR REFERENCE TO ANTIBIOTIC PRODUCTION

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A further characterization of *Actinomyces tumemacerans* KRASSILNIKOV and KOVESHNIKOV, 1962 strain INMI P-42 was carried out. It is classified in Section *Monoverticillus-Spira*, Series white to gray. When the organism was shake-cultured in a wheat flour medium, an antibacterial antibiotic with limited antitumor activity was isolated from the culture filtrate and a tetraene antibiotic from the mycelial cake. The antibacterial antibiotic proved to be closely related to or identical with BA-180265 A (kanchanomycin) reported by LIU *et al.* in 1963.

A strain designated INMI P-42 was isolated from a soil sample from Askania Nova in the Ukrainian SSR and proposed as a new species *Actinomyces tumemacerans* by KRASSILNIKOV and KOVESHNIKOV in 1962[1], because of its activity on plant tumors caused by *Agrobacterium tumefaciens*. Since then, its secondary metabolites have been studied and characterized by Russian scientists[2,3]. The antibiotics produced were named P-42A, P-42B, P-42E and P-42S. None of them have been obtained in pure state or adequately identified as yet. Using paper chromatographic methods, TOKHTAMURATOV and SILAEV[3] concluded that strain INMI P-42 produces four antibiotics similar to nystatin (P-42B), rimocidin (P-42E), cycloheximide (P-42A) and streptomycin or kanamycin (P-42S), when the organism was incubated in fish extract medium containing glucose.

Subsequently, MAEVSKY and one of the present authors[4] recognized that strain INMI P-42, when incubated in a wheat flour medium, also produces activity against EHRLICH ascites tumors and leukemia L-1210 *in vivo*.

In this paper, comparative studies of strain INMI P-42 with known streptomycetes and further purification and identification of the antibiotic active against animal tumors *in vivo* are discussed.

Experimental

A. Characterization of *Actinomyces tumemacerans* KRASSILNIKOV and KOVESHNIKOV, Strain INMI P-42

1. Morphology.

Strain INMI P-42 produces primary verticils with open coils as shown in Plate 1.

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Plate 1. Morphology of spore chain of *Actinomyces tumemacerans* strain INMI P-42

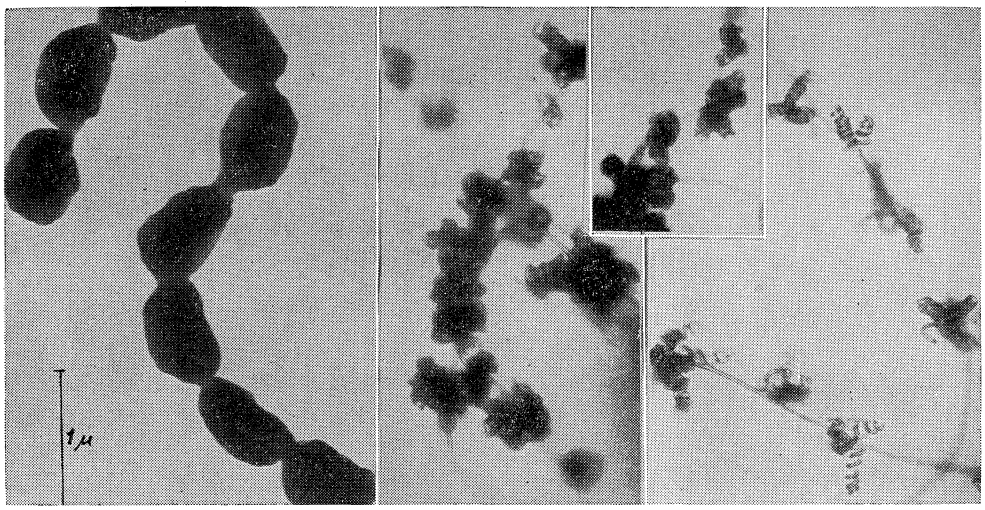


Table 1. Cultural characteristics of *Actinomyces tumemacerans* strain INMI P-42

Medium	Growth	Aerial mycelium	Vegetative mycelium	Soluble pigment
Yeast extract agar	Abundant	Scant, grayish white (5 ba)	Straw yellow to brown (2 ic)	None
Starch asparagine agar	Poor	None	Punctiform, white	None
Egg medium	Abundant	Scant, powdery, white (3 cb)	Much wrinkled, brownish straw to cinnamon (3 pg)	None
Calcium hydroxysuccinate agar	Moderate	Moderate, powdery, ash gray with brownish eucalyptus green tinge (2 dc)	Cream white to bronze brown (1 ic)	Scant, greenish
Glucose bouillon	Abundant	Scant, dull white	Membranous and flocculant, creamy white	None
Tyrosine agar	Fair	None	Minute colonies, spreading, cream white with brownish tinge	Faint, yellowish

( ): Delineated by color wheels made with tabs from IVth eddition of the Color Harmony Manual.

Accordingly, it would be categorized in Section *Monoverticillus-Spira* PRIDHAM *et al.*[5] Typical morphology of sporophores was observed on glucose-asparagine agar and modified chitin medium[6]. The spores are short and cylindrical ($0.45 \times 0.7\ \mu$). Their surfaces are smooth with some irregularities when examined under the electron microscope.

2. Cultural and physiological characteristics.

Growth characteristics of the strain on yeast extract agar, egg medium, calcium hydroxysuccinate agar, glucose bouillon and tyrosine agar[7] are summarized in Table 1. The color names described were taken from the Color Harmony Manual[8] and the Dictionary of Color[9]. Growth was moderate to abundant depending on the medium used. Vegetative mycelium was white, later becoming brownish cream on almost all media. A purplish or greenish tinge developed in vegetative mycelium growing on some chemically-defined media.

The aerial mycelium formed was scant and white on most media except for calcium hydroxysuccinate agar, on which it is moderate, powdery and grayish white

with greenish tinge.

A slight yellowish brown soluble pigment was produced with some chemically-defined media.

The physiological properties of strain INMI P-42 are shown in Table 2. Nitrate reduction, blood haemolysis and serum liquefaction were positive. Diastatic action, cellulose decomposition and melanin formation were negative. The media used for these tests were listed in the table.

Table 2. Physiological characteristics of *Actinomyces tumemacerans* strain INMI P-42

Activity	Result	Medium
Nitrate reduction	Positive	Medium for nitrate reduction
Haemolysis	Positive	Blood agar
Serum liquefaction	Positive	LÖFFLER'S serum medium
Diastatic action	Negative	Starch agar
Cellulose decomposition	Negative	Cellulose medium
Melanin formation	Negative	Tyrosine agar

3. Comparison of strain INMI P-42 with known *Streptomyces* species.

Streptomyces or *Streptoverticillia* classified in Section *Monoverticillus-Spira* are *Streptomyces echinatus*, *S. netropsis*, *S. circulatus*, *S. matensis*[10] and *Streptoverticillium rubrireticuli* var. *pimprina*[11].

Streptomyces echinatus was classified in Section *Monoverticillus-Spira* by WAKSMAN[10]. However, LOCCI *et al.*[12] noted that the micromorphology of this species is clearly pseudoverticillate and not verticillate. This also was the case in our own observation of *S. echinatus* strain IFM 1076. *S. echinatus* is further differentiated from strain INMI P-42 in its yellow to greenish yellow growth and soluble pigment, and its ash gray aerial mycelium.

Streptomyces netropsis differs clearly from strain INMI P-42 in its production of red aerial mycelium, spore chains with curling tips and brown soluble pigment on proteinaceous media.

Streptoverticillium rubrireticuli var. *pimprina* was described as being biverticillate with spiralled spore chains, but the illustration showed it to be monoverticillate with coils. On the other hand, the vegetative growth contains patches with a reddish tinge owing to the presence of the reddish antibiotics (streptorubins A and B).

The growth characteristics of strain INMI P-42 were further compared with the descriptions of *S. circulatus* and *S. matensis* as shown in Table 3.

Table 3. Comparison of growth characteristics of *Actinomyces tumemacerans* strain INMI P-42 with *Streptomyces circulatus* and *Streptomyces matensis*

Property	*A. tumemacerans*	*S. circulatus*	*S. matensis*
Color of vegetative mycelium	White to brownish cream with purplish tinge	Colorless	Colorless to purplish
〃 aerial mycelium	White or grayish	White	Whitish to light gray
〃 soluble pigment	Slight, yellowish brown	No description	Faint bluish
Calcium hydroxysuccinate agar			
VM	Moderate, bronze brown	No description	Poor
〃 AM	Moderate, ash gray	No description	None
〃 SP	Scant, purplish	No description	No description
Melanin formation	Negative	No description	Said to be positive
Antagonistic property	The crystalline antibiotic	Limited	Matamycin

Streptomyces circulatus is weakly proteolytic and shows limited antagonistic activity and produces abundant aerial mycelium on chemically-defined media. Furthermore, HÜTTER in 1967[13] and LOCCI *et al.*[12] reexamined *S. circulatus* and stated that the species does not produce true verticils.

Streptomyces matensis is differentiated from strain INMI P-42 by its weakly proteolytic activity, pinkish to violet-gray reverse color and gray aerial mycelium on chemically-defined media, poor growth without aerial mycelium and production of faint bluish soluble pigment on calcium hydroxysuccinate agar, and production of the antibiotic matamycin. On the other hand, strain INMI P-42 produces brownish cream reverse color with purplish or greenish tinge, slightly yellowish brown soluble pigment, no melanin pigment, and a yellowish orange colored antibiotic which is active against bacteria, yeasts, HeLa cell cultures and EHRLICH ascites tumor *in vivo*.

Therefore, we agree with KRASSILNIKOV and KOVESHNIKOV's proposal that strain INMI P-42 represents another species of *Actinomyces*.

B. Isolation and Characterization of the Antibiotics from Strain INMI P-42

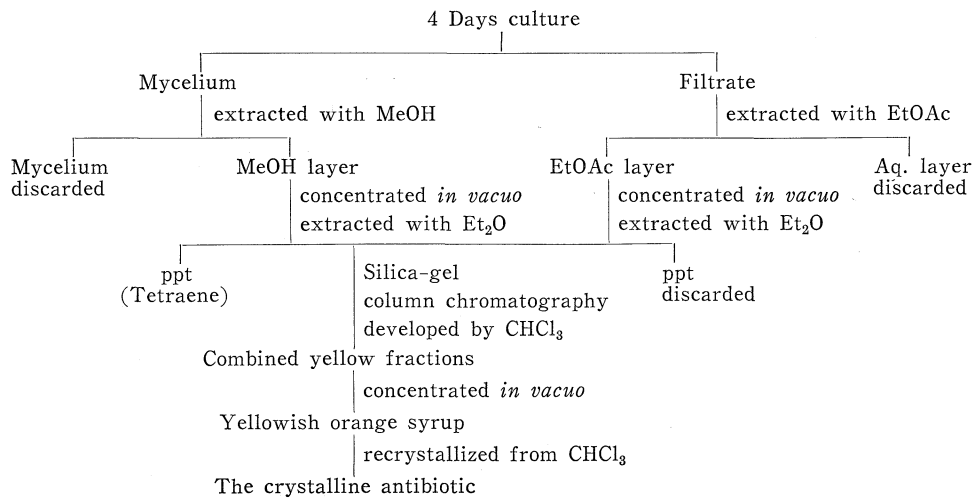
Strain INMI P-42 was shake-cultured in flour medium containing 20 g of wheat flour, 0.05 g of yeast extract and 0.5 g of Polypeptone in 1 liter of tap water (pH 7.4).

Incubation was carried out at 27°C for 4 days. When 0.25 ml/mouse/day of the culture filtrate was administered intraperitoneally to mice with EHRLICH ascites tumors starting 24 hours after implantation and continuing for 7 successive days, prolongation of life span without increase in body weight was observed. The culture filtrate also exhibited antibacterial, antifungal and anti-HeLa cell activities.

A flow sheet for the isolation and purification of the antibacterial and anti-tumor antibiotic of strain INMI P-42 is shown in Fig. 1.

Methanol extracts of mycelia did not show any activity against EHRLICH ascites tumor *in vivo*, but were active against HeLa cell cultures, and fungi. Only slight activity was noted against bacteria. The principal antibiotic isolated from this mycelial extract was an antifungal tetraene. It was not further characterized and

Fig. 1. Extraction flow sheet



its relationship to antibiotics P-42B and P-42E is not known. The ether-soluble fraction of the mycelial extract was combined with that of the culture filtrate.

The main active fraction of the culture filtrate was extracted with ethyl acetate at pH 7. The yellow solvent layer was concentrated *in vacuo* and again extracted with ether.

The ether-soluble fractions were combined and further purified by chromatography on a silica gel column. By developing the column with chloroform, active fractions pigmented yellow were eluted. These fractions were combined and concentrated, and the resulting yellowish orange crystalline antibiotic was recrystallized from chloroform. All fractions other than the ether and chloroform-soluble fractions were inactive against EHRLICH ascites tumor *in vivo*, although some of them showed antimicrobial activity. The antifungal tetraene also was obtained from these fractions. It was not further characterized.

The purity of the crystalline antibiotic was determined by thin-layer chromatography employing five solvent systems. Only a single spot was detected with each solvent system. As summarized in Table 4, the Rf values were 0.73, 0.65, 0.46, 0.1 and 0.0 in acetone - methanol - acetic acid (1 : 1 : 1), 1-butanol - methanol - 10 % citric acid

Table 4. Paperchromatographic characterization of the crystalline antibiotic

Solvent system	Rf value
Acetone - methanol - acetic acid (1 : 1 : 1)	0.73
1-Butanol - methanol - 10% citric acid (4 : 2 : 2)	0.65
1-Butanol - acetic acid - methanol (3 : 1 : 1)	0.46
Benzene - ethyl acetate - methanol (10 : 2.5 : 1)	0.1
Water	0.0

Fig. 2. Ultraviolet absorption spectra of the crystalline antibiotic and BA-180265 A (in MeOH)

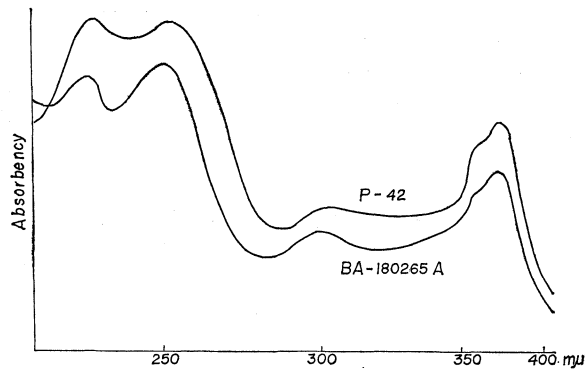
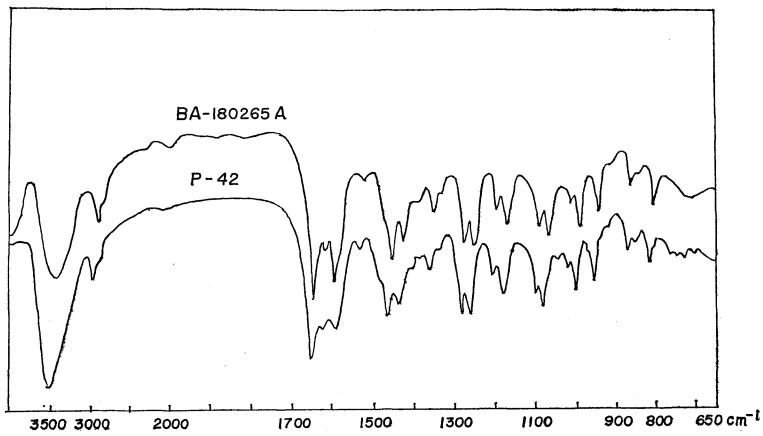


Fig. 3. Infrared absorption spectra of the crystalline antibiotic and BA-180265 A (KBr disc.)



(4 : 2 : 2), 1-butanol - acetic acid - methanol (3 : 1 : 1), benzene - ethyl acetate - methanol (10 : 2.5 : 1) and water, respectively.

Interpretation of the ultraviolet and infrared absorption spectra (Figs. 2 and 3) suggested that the crystalline antibiotic was closely related to BA-180265 A (kanchanomycin; IHNDRIS[14]) isolated by LIU *et al.*[15] and RAO *et al.*[16] from an unidentified streptomycete.

Physicochemical properties were compared with those of BA-180265 A as shown in Table 5. Some differences in physicochemical properties were observed.

Table 5. Physicochemical properties of the crystalline antibiotic and BA-180265 A

Property	The crystalline antibiotic	BA-180265 A
M. P. (°C)	250~255	265~268
$[\alpha]_D$ ($CHCl_3$)	−300°	−634° *, −585° **
UV max (mμ)	229, 253, 305, 375	231, 252, 300, 370
Crystal	Yellowish orange prism	Bright yellow prism
Soluble in	Me_2CO, $CHCl_3$, Et_2O, MeOH	Me_2CO, $CHCl_3$, Et_2O, MeOH
Insoluble in	H_2O	H_2O
Color change with conc. H_2SO_4	Orange-brown to green	Orange-brown to green
conc. HNO_3	Purplish-red	Purplish-red
$FeCl_3$	Deep green	Deep green

*: Recrystallized from acetone. **: Recrystallized from chloroform.

The melting point of the crystalline antibiotic is 250~255°C after repeated recrystallization while that of BA-180265 A is 265~268°C. Specific rotation in chloroform is −634° and −585° for BA-180265 A recrystallized from acetone and from chloroform respectively, but for the crystalline antibiotic, it is −300°. The solubility of both antibiotics is about the same. The present antibiotic gives an orange brown color with concentrated nitric acid and a deep green color with alcoholic ferric chloride. These reactions are identical with those described for BA-180265 A.

Table 6. Biological activities of the crystalline antibiotic and BA-180265 A

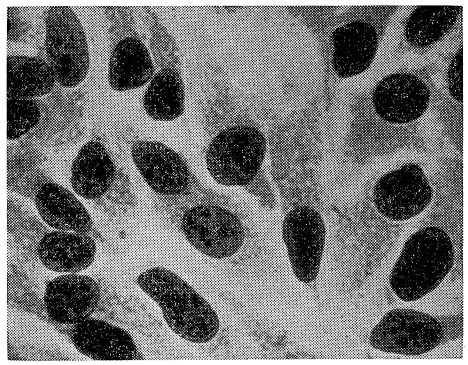
Activity against	The crystalline antibiotic	BA-180265 A
Staphylococcus aureus	1~0.0078 incomplete	0.0005
Bacillus subtilis PCI 219	0.25	No description
Candida albicans	0.0078	0.0002
Aspergillus niger	>1.0	No description
HeLa cell culture	0.1 desquamation 0.05 cytotoxicity	Lethal activity at 0.005~0.01
EHRLICH ascites tumor	10.0~50.0 *	Marginal activity

Number indicates minimum inhibition concentration in mcg/ml.
*: Effective dose in mcg/ml (0.25 ml/day/mouse for 7 days intraperitoneally).

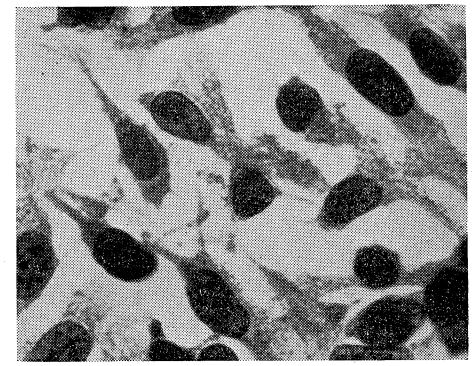
The LD_{50} for the crystalline antibiotic was found to be 375 mcg/kg of body weight when given to mice intravenously, but mice tolerated 12.5 mg/kg by intraperitoneal administration.

Biological activities of the crystalline antibiotic and BA-180265 A are compared in Table 6. LIU *et al.*[15] and RAO *et al.*[16] reported that BA-180265 A is active against *Staphylococcus aureus* at a concentration of 0.0005 mcg/ml and against *Candida albicans* at 0.0002 mcg/ml. On the other hand, the minimal inhibitory concentration of the crystalline antibiotic was 0.0078 mcg/ml against *Candida albicans* strain YU-1200 and

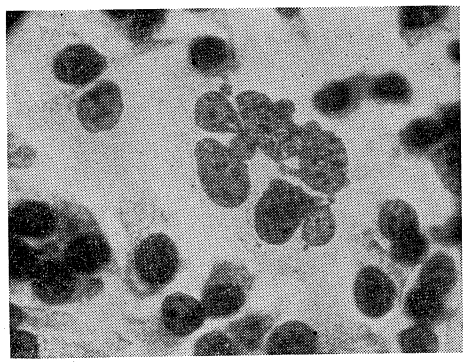
Plate 2. Cytotoxicity of the crystalline antibiotic (0.005 mcg/ml)



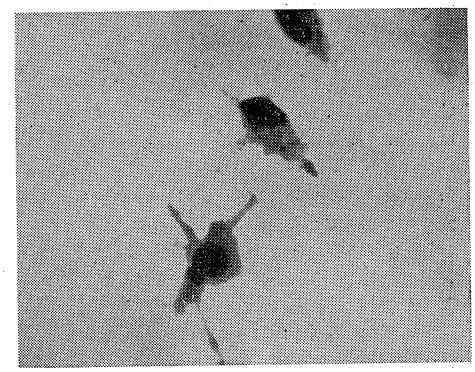
Normal



Elongation



Bizarre nuclei



Desquamation

Staphylococcus aureus strain FDA 209 P was incompletely inhibited in the range from 0.0078 to 1.0 mcg/ml. The crystalline antibiotic also inhibited *Bacillus subtilis* strain PCI 219 in a concentration of 0.25 mcg/ml but was not inhibitory to *Aspergillus niger* in a concentration of 1.0 mcg/ml.

BA-180265 A is reported to show lethal activity in concentration from 0.005 to 0.01 mcg/ml against HeLa cell cultures and only marginal activity against the standard mouse and rat tumors. Our crystalline antibiotic exhibited anti-tumor activity *in vivo* when 2.5~12.5 mcg/mouse/day was administered to mice with EHRLICH ascites tumor. HeLa cell cultures were desquamated at a concentration of 0.1 mcg/ml; and at a concentration of 0.05 mcg/ml cytotoxicities such as desquamation, roundation, elongation and bizarre nuclei were observed as shown in Plate 2.

Discussion and Conclusion

In 1962, KRASSILNIKOV and KOVESHNIKOV described *Actinomyces tumemacerans* which produces antibiotic active against plant tumors. They reported the formation of druses on the aerial mycelium.

The results of the present experiments have shown that these druses are the conglomeration of spiralled verticils which are attached to long, straight branches. Additional information on the mycological aspects of *A. tumemacerans* is given and the strain is classified in Section *Monoverticillus-Spira*, Series white to gray.

The principal antibiotic elaborated in the culture filtrate when the strain was shake-cultured in wheat flour medium was identified as kanchanomycin (BA-180265 A) like compound on the basis of its physicochemical and biological properties. It is possible that the discrepancy between our results and those of LIU *et al.*[15] lies in the purity of both preparations. The strain also produces a tetraene antibiotic (not further characterized) in the mycelium.

References

1) KRASSILNIKOV, N. A. & A. D. KOVESHNIKOV : *Actinomyces tumemacerans* n. sp. A new species inducing disintegration of tumors in plant. Mikrobiologii 31 : 589~594, 1962
2) TOKHTAMURATOV, E.; A. B. SILAEV & S. M. KHODZHIBAEVA : Isolation of an antitumor substance from the culture fluid of *Actinomyces tumemacerans* P 42. Antibiotiki 9 : 205~208, 1964
3) TOKHTAMURATOV, E. & A. B. SILAEV : Recovery and purification of antibiotics produced by *Act. tumemacerans*. Antibiotiki 10 : 30~33, 1965
4) MAEVSKY, M. M. & T. F. KUIMOVA : Unpublished data.
5) PRIDHAM, T. G.; C. W. HESSELTINE & R. G. BENEDICT : A guide for classification of *Streptomyces* according to selected groups. Appl. Microbiol. 6 : 52~79, 1958
6) CAMPBELL, L. L. & O. B. WILLIAMS : A study of chitin-decomposing micro-organisms of marine origin. J. Gen. Microbiol. 5 : 894~905, 1951
7) SHIRLING, E. B. & D. GOTTLIEB : Methods for characterization of *Streptomyces* species. Intern. J. Systemat. Bacteriol. 16 : 313~340, 1966
8) JACOBSON, E.; W. C. GRANVILLE, C. E. FOSS & R. E. CKERSTROM : Color Harmony Manual. Container Corporation of America, Chicago, 1958
9) MAERZ, A. & M. R. PAUL : A dictionary of color. 2nd Ed., McGraw-Hill Book Co., Inc., U.S.A., 1950
10) WAKSMAN, S. A. : The Actinomycetes. Vol. 2, The Williams & Wilkins Co., Baltimore, U. S. A, 1961
11) THIRUMALACHAR, M. J.; N. V. BRINGI, P. V. DESHMUKH, P. W. RAHALKAR, R. INDIA & K. S. GOPALKRISHNAN : Streptorubin A and B. New antibiotics with cytotoxic properties. Hindustan Antibiot. Bull. 7 : 18~24, 1964.
12) LOCCI, R.; E. BALDACCI & B. P. BALDAN : The genus *Streptoverticillium*. A taxonomic study. Giorn. Microbiol. 17 : 1~60, 1969
13) HÜTTER, R. : Systematik der Streptomyceten unter besonderer Berucksichtigung der von ihnen gebildeten Antibiotica (Bibliotheca Microbiologica, Fasc. 6). S. Karger AG, Basel, # pp. 110~138, 1967
14) IHNDRIS, R. W. : Synonymus of cancer chemotherapy agents. Appendix II. Compounds tested clinically as anticancer agents. Cancer Chemotherapy Rept. 28 : 67~73, 1963
15) LIU, WEN-CHIN; W. P. CULLEN & V. RAO : BA-180265 : a new cytotoxic antibiotic. Antimicr. Agents & Chemoth. -1962 : 767~771, 1963
16) RAO, V. K.; P. BROOK, W. S. MARSH, WANAQUE & WEN-CHIN LIU : Antibiotic complex BA-180265 (A, B) and process for making same. U. S. Patent 3,285,814. Nov. 15, 1966