AURANTININ, A NEW ANTIBIOTIC OF BACTERIAL ORIGIN

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A new polyene class antibiotic, aurantinin (KM-214), was isolated from the fermentation broth of *Bacillus aurantinus* MASUMA and ŌMURA sp. nov. The substance is a conjugated triene with a molecular weight of 618 and molecular formula $C_{35}H_{54}O_9$, and melts at 139~140°C. The antibiotic is active *in vitro* against Gram-positive bacteria, but not against yeast and fungi.

During the course of our screening for new antibiotics, a bacterial strain No. KM-214 isolated from a soil sample collected at Ueno, Tokyo, Japan was found to produce an antibiotic.[1] An aurantinin-producing organism designated as *Bacillus aurantinus* MASUMA and ŌMURA sp. nov. is described. The morphological, cultural and physiological characteristics of the organism as well as its taxonomic relationship to other species of *Bacillaceae* are discussed. The type strain is KM-214 by virtue of its being a single isolate. Isolation and purification of the antibiotic gave an amorphous light yellow powder. As the result of comparative studies of the antibiotic with other known triene antibiotics,[2~8] it was shown to be a new antibiotic and named aurantinin. The present paper describes the morphological characteristics of the strain, the fermentation, isolation and properties of the new antibiotic in detail.

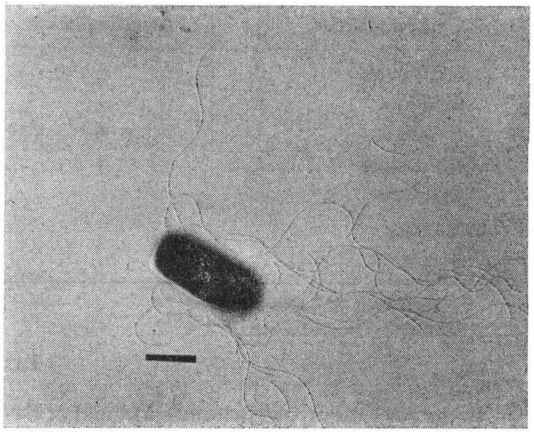
Morphological Characteristics

For the characterization of strain KM-214, the procedures described by GIBBS & SKINNEW[9], GIBBS & SHAPTON[10], and IIZUKA & SETO[11] were followed. For identification of the organism, the descriptions included in BUCHANAN *et al.*[12] and SKERMAN[13] were used.

Morphological observation of strain No. KM-214 was carried out by both optical and electron microscopy with cells cultured mainly on nutrient agar for 18~24 hours at 37°C. The following results were obtained. The culture consists of rods with rounded ends, 0.7~1.0×2~4 μ, occurring singly or sometimes in pairs. The strain is motile, possessing peritrichous flagella. It is Gram-positive to variable, not acid-fast stained and has central spore.

A typical electron-microscopic photograph of strain No. KM-214 having flagella is shown in Fig. 1.

Fig. 1. Electron microscopic photograph of *B. aurantinus*. A mark equals 1 μ.



Culture Characteristics

Observation of strain KM-214 was carried out using cultures grown on various media for 2~10 days. The following results were obtained.

1) Colonies on nutrient agar plates (for 2 days at 37°C): 0.5~2.0 mm in diameter, circular, capitate, entire, amorphous type with smooth surface and opaque, creamy white.

2) Nutrient agar slants (for 2 days at 37°C): Moderate growth, capitate raised in center, smooth surface, glistening opaque and creamy white color.

3) Nutrient broth (for 2 days at 37°C): Moderate growth, membranous, slightly turbid, compact and pale yellow color.

4) Potato plug (for 1 day at 37°C): Abundant growth, smooth and soft to slimy, yellow orange color. No soluble pigment.

5) Litmus milk (for 10 days at 37°C): Moderate growth, membranous. Positive peptonization without coagulation, an alkaline reduction of litmus.

6) Gelatin stab (for 10 days at 20°C): Moderate growth, liquefaction in infundibular form, no soluble pigment.

Physiological Characteristics

The physiological characteristics of strain KM-214 are summarized in Tables 1 and 2.

Table 1. Physiological properties of *B. aurantinus*.

VP test	+	Tyrosine decomposition	−
MR test	+	Catalase	+
Indole formation	−	O-F test (substrate: glucose)	+
H_2S formation	−	Anaerobic growth	−
KNO_3 reduction	+	NaCl (15%)	−
Citrate utilization	+	Temp. for growth	5~45°C
Starch hydrolysis	+	Opt. temp.	25~37°C
Gelatin hydrolysis	+	pH for growth	5~8
Casein decomposition	+	Opt. pH	7

+: positive, −: negative

Table 2. Cleavage of carbohydrate by *B. aurantinus*.

Carbon source	Acid formation	Carbon source	Acid formation
L-Arabinose	+	D-Sorbitol	+
D-Xylose	+	D-Mannitol	+
D-Glucose	+	Inositol	+
D-Fructose	+	Glycerol	+
Maltose	+	Starch	+
Sucrose	+	D-Mannose	−
Lactose	+		

+: positive, −: negative

Taxonomy

In the 8th Edition of "BERGEY's Manual of Determinative Bacteriology"[12], five genera of the Family

Table 3. Comparisons of seven *Bacillus* species and *B. aurantinus*

	Spore distends			Products of growth on glucose			Starch hydrolysis	Nitrite from nitrate	Growth in anaerobic agar	Tyrosine decomposed	Acid from arab. xyl. mannit.
	Shape	Sporangium distinctly	Dominant position	Acid	Gas	Acetoin					
B. subtilis	E	—	C	+	−	+	+	+	−	−	+
B. pumilus	E	−	C	+	−	+	−	−	−	−	+
B. licheniformis	E	−	C	+	w or −	+	+	+	+	−	+
B. cereus	E	−	C	+	−	+		+	+	+	−
B. anthracis	E	−	C	+	−	+		+	+	d	−
B. thuringiensis	E	−	C	+	−	+		+	+	d	−
B. megaterium	E	−	C	+	−	−		d	−	d	d
B. aurantinus	E	−	C	+	−	+	+	+	−	−	−

The symbols used are: E, elliptical or cylindrical; C, central; +, positive; −, negative; w, weakly positive; d, reactions differ.

Bacillaceae are described, among which three, *i. e.*, *Sporosarcina, Clostridium* and *Desulfatomaculum* are clearly differentiated from strain KM-214. Furthermore, the genus *Sporolactobacillus* does not produce catalase. Therefore the present organism does not belong to these four genera. However, the Genus *Bacillus* (CONN, 1872) includes soil organisms and has cells which are aerobic or facultative and they usually produce catalase. These properties are in accordance with those of strain KM-214. Therefore we assigned it to the genus *Bacillus*.

Strain KM-214 produces acid and acetoin on media containing glucose. Seven species of the genus *Bacillus* having such abilities are described in BERGEY's manual. The characteristics of strain KM-214 and those species are compared in Table 3.

The results indicate that strain KM-214 has some characteristics in common with *B. subtilis* or *B. pumilus*, but differs from *B. subtilis* in the culture characteristics on nutrient agar plate, *i.e.* colonies of *B. subtilis* are rosulate, undulated and spreading, but those of the strain are circular, entire and not spreading, moreover the strain produces yellow-orange pigment on potato plug and a new antibiotic, aurantinin. Furthermore, *B. pumilus* has the differences in chromogenicity, starch hydrolyzation, reduction of nitrate, milk peptonization.

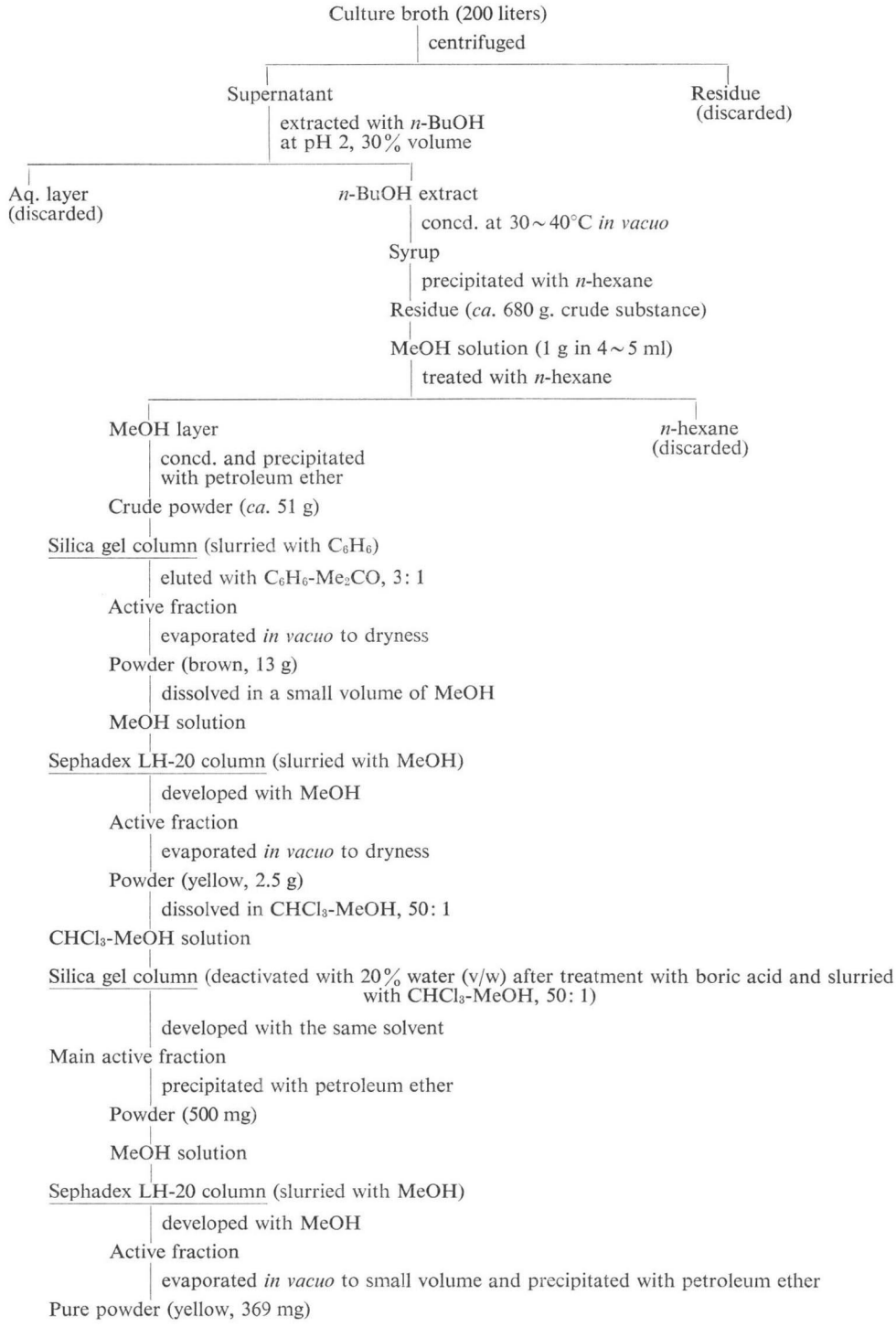
The various morphological, cultural and physiological characteristics of strain KM-214 described above lead us to conclude that strain KM-214 is a new species of *Bacillus*, for which the name *Bacillus aurantinus* MASUMA and ŌMURA sp. nov. is proposed.

The epithet aurantinus is derived from the color of the growth on potato plug media. The type strain has been deposited in the collection of the Fermentation Research Institute, Agents Industrial Science and Technology, Tokyo and assigned the designation FERM-P No. 3379.

Fermentation

A well-grown agar slant of the KM-214 producing organism was used to inoculate into the seed medium containing 1.0% glycerin, 1.0% starch, 2.0% soybean meal, 0.3% dried yeast, 0.5% NaCl, 0.2% $(NH_4)_2SO_4$, 0.1% K_2HPO_4 and 0.3% $CaCO_3$, the pH being adjusted to 7.0 before sterilization. The seed culture was incubated at 27°C for 24 hours on a reciprocal shaker (120 rpm), and 200 ml of the seed was transferred into 20-liters of the same medium in a 30-liter jar fermentor. Antibiotic concentration

Scheme 1. Isolation and purification procedure for aurantinin

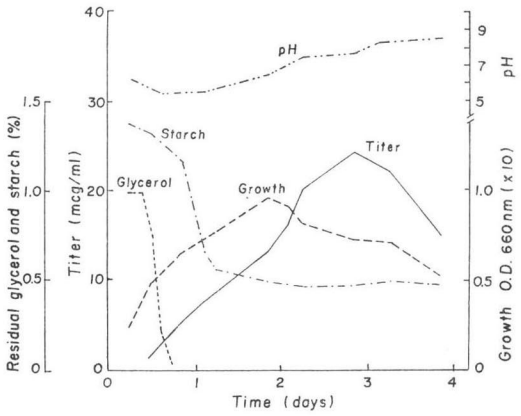


reached a maximum after 2~3 days at 27°C. The time course of the jar fermentation is shown in Fig. 2.

The antibiotic activity in the fermentation broth was determined by a paper disc-agar diffusion assay using *Clostridium perfringens* ATCC 3624. In the jar fermentation, strain KM-214 produced titres of 20~30 mcg/ml.

Fig. 2. Time course of aurantinin production.

Cultivation was performed using a 30-liter jar fermentor containing 20 liters of the medium as described in text. Culture conditions were as follows; temp. 27°C, agitation 250 rpm and aeration 0.5 vol/vol/min.



Isolation and Purification

The isolation and purification of aurantinin were carried out by the procedure shown in Scheme 1.

The antibiotic has an Rf value of 0.35 when subjected to tlc on Merck silica gel, using chloroform - methanol mixture (8: 1, v/v) for development. The average yield of the antibiotic was 1.8 mg per liter of culture broth.

Physico-chemical Properties

Aurantinin is an amorphous yellow powder which is readily soluble in methanol, ethanol, ethyl acetate, acetone and dioxane, slightly soluble in chloroform and ethyl ether, and practically insoluble in petroleum ether, *n*-hexane and water. It gives positive reactions with potassium permanganate and sulfuric acid (purple), but is negative in the ferric chloride, ninhydrin, as well as LASSAIGNE, BEILSTEIN and MOLISCH reactions.

Aurantinin melts at 139~140°C, $[\alpha]_D^{22}+126°$(*c* 1.0, MeOH), analyzed for $C_{35}H_{54}O_9$. MS *m/e* 600.361 (M^+-18, calcd. for $C_{35}H_{52}O_8$).

Anal. Calcd.: C 67.93, H 8.80
Found: C 68.00, H 8.16, N 0.00

No sulfur and halogen can be detected in aurantinin preparations.

It gave a di-O-acetate, prepared with acetic anhydride and pyridine, mp 103~105°C (decomp.), $[\alpha]_D^{35}+155°$ and analyzed for $C_{39}H_{58}O_{11}$. MS *m/e* 702.392 (calcd. for $C_{39}H_{58}O_{11}$).

Anal. Calcd.: C 66.67, H 8.26
Found: C 67.12, H 7.75

The diacetyl monomethyl ester was prepared, with diazomethane in methanol, mp 64~68°C, and analyzed for $C_{40}H_{60}O_{11}$. MS *m/e* 716.405 (calcd. for $C_{40}H_{60}O_{11}$).

Anal. Calcd.: C 67.04, H 8.38
Found: C 67.87, H 8.04

The UV spectra of aurantinin in methanol (Fig. 3) shows presence of a triene with maxima at 268 (sh), 278, 287 (sh) nm and with $E_{1cm}^{1\%}$ of 500, 630 and 580, respectively. The molecular extinctions calculated for a molecular weight of 618 are $\varepsilon_{268}=30{,}900$, $\varepsilon_{278}=38{,}900$ and, $\varepsilon_{287}=35{,}800$, respectively. These values are very close to those given in the literature for trienic hydrocarbons[14]. As calculated from the molecular extinctions at 278 nm of *trans* (C4)-*trans* (C6) alloocimene and[14] aurantinin, the

Fig. 3. UV spectra of aurantinin (MeOH, 10 mcg/ml).

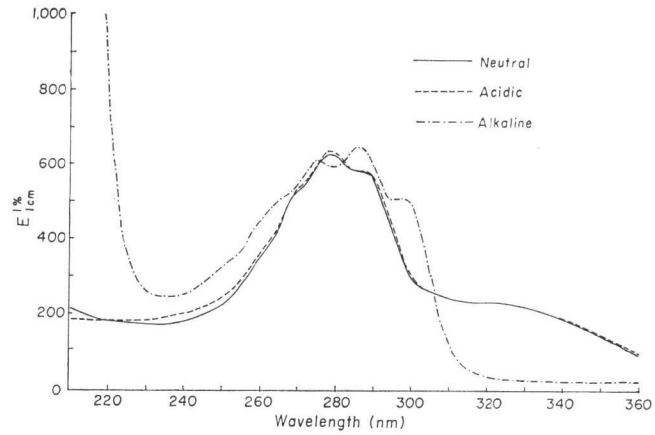


Fig. 4. IR spectrum of aurantinin (KBr).

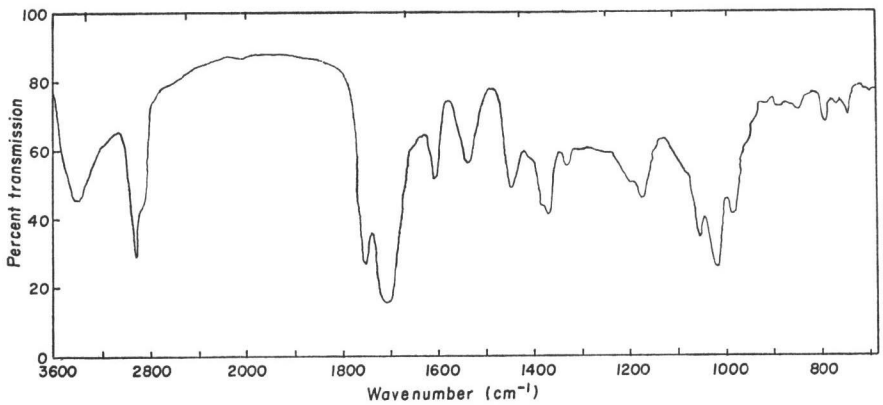
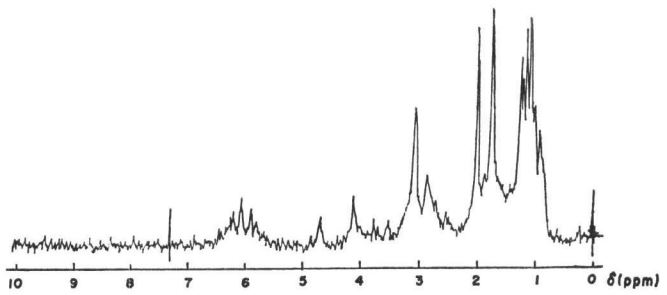


Fig. 5. NMR spectrum of aurantinin ($CDCl_3$, 100 MHz).

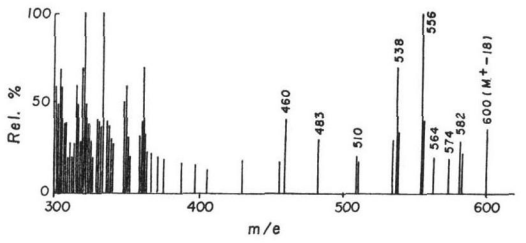


equivalent weight of aurantinin is about 630.

The IR spectrum (Fig. 4) of aurantinin shows the presence of OH functions (wide band between 3500 and 3300 cm^{-1}), lactone (1750 cm^{-1}) and carbonyl (1710 cm^{-1}) groups. Absorption at 993 cm^{-1} is due to the triene system[8,9].

The NMR spectrum ($CDCl_3$, 100 MHz) of the antibiotic (Fig. 5) shows two singlets at 2.0 and 1.8 ppm due to an acetyl group. Broad peaks are observed at 5.7 and 6.4 ppm due to the protons on the ole-

Fig. 6. Mass spectrum of aurantinin.



finic carbon positions. No aromatic hydrogen absorptions are present.

Lastly, the mass spectrum of aurantinin (Fig. 6) shows a fragmentation pattern in which the molecular ion peak is not observed. The peak at *m/e* 600 is due to the loss of a water molecule from the parent ion since molecular ion peaks at *m/e* 702 and 716 are found with its diacetate and diacetylmonomethyl ester, respectively.

On the basis of its ultraviolet absorption, aurantinin can be classified as a triene, but comparison with other known trienes shows it to be a novel compound.

Biological Properties

The antimicrobial spectrum of aurantinin has been determined by the agar dilution method. The antibiotic was effective against Gram-positive bacteria including anaerobic bacteria but inactive against Gram-negative bacteria, filamentous fungi and yeasts. The minimum inhibitory concentrations (MIC) against a variety of microorganisms are shown in Table 4.

No acute toxicity of the antibiotic was observed in mice after intraperitoneal injection of 96 mg/kg and oral administration of 335 mg/kg.

Table 4. Antimicrobial spectrum of aurantinin.

Test organism	MIC* (mcg/ml)
Clostridium perfringens ATCC 3624	1.56
Clostridium perfringens PB6K N5	12.5
Clostridium botulium IFO 3733	0.4
Clostridium kainantoi IFO 3353	0.4
Clostridium sporogens IFO 3987	0.78
Staphylococcus aureus FDA 209P	0.2
Staphylococcus aureus JC-1	0.78
Staphylococcus aureus FS 1277	0.78
Staphylococcus albus	0.78
Bacillus subtilis PCI219	25
Bacillus cereus IFO 3001	0.78
Bacillus cereus var. *mycoides*	0.78
Bacillus agri	1.56
Bacillus anthracis	3.12
Sarcina lutea ATCC 9341	25
Mycobacterium smegmatis ATCC 607	100
Nocardia asteroides	25
Corynebacterium paurometabolum	1.56
Escherichia coli NIHJ	>100
Escherichia coli NIHJ JC-2	>100
Klebsiella pneumoniae PCI 602	>100
Salmonella typhimurium	>100
Proteus mirabilis IFO 3849	>100
Proteus morganii IFO 3168	>100
Pseudomonas aeruginosa P-3	>100
Pseudomonas aeruginosa GN-918	>100
Aerobacter aerogenes ATCC 9621	>100
Bordetella bronchiseptica	>100
Xanthomonas oryzae	3.12
Candida albicans	>100
Saccharomyces sake	>100
Aspergillus niger	>100
Aspergillus brevipus	>100
Aspergillus fumigatus	>100
Trichophyton interdigitale	>100
Trichophyton roseum	>100
Trichophyton mentagrophytes	>100
Penicillium chrysogenum	>100
Alternaria kikuchiana	>100
Sclerotinia cinerea	>100

* Agar dilution method

Discussion

The production of polyene antibiotics by the genus *Streptomyces* is of widespread occurrence. However, with the exception of several trienes[8,18], most polyenes so far described possess four to seven conjugated double bonds. Generally polyenic antibiotics are fairly toxic products, rather unstable and active only on yeasts and fungi. Aurantinin is the first antibiotic containing a conjugated triene system to be isolated from a culture of the genus *Bacillus* and it is active against Gram-positive bacteria and not yeasts and fungi. A similar antibiotic, resistaphylin[10,15], has been reported to be a

triene antibiotic and is also very active against Gram-positive bacteria. However, resistaphylin has UV absorption at 230 nm and contains nitrogen whereas aurantinin does not.

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References

1) ŌMURA, S.; T. NISHIKIORI, R. ŌIWA, Y. IWAI, R. MASUMA & M. KATAGIRI: A new antibiotic KM-214 produced by a strain of *Bacillus*. J. Antibiotics 29: 477~478, 1976
2) ARMSTRONG, J. J.; J. F. GROVE, W. B. TURNER & G. WARD: An antifungal triene from a *Streptomyces* sp. Nature 206: 399~400, 1965
3) CORONELLI, C.; R. C. PASQUALUCCI, J. E. THIEMANN & G. TAMONI: Mycotrienin, a new polyene antibiotic isolated from *Streptomyces*. J. Antibiotics, Ser. A 20: 329~333, 1967
4) ASZALOS, A.; R. S. ROBISON, P. LEMANSKI & B. BERK: Trienine, an antitumor triene antibiotic. J. Antibiotics 21: 611~615, 1968
5) AIZAWA, S.; M. SHIBUYA & S. SHIRATO: Resistaphylin, a new antibiotic. I. Production, isolation and properties. J. Antibiotics 24: 393~396, 1971
6) POLTORAK, V. A.; Z. G. SOKOLOVA, K. A. VINOGRADOVA & A. B. SILAEV: Triene, an antifungal antibiotic, its isolation and properties. Antibiotiki 17: 738~742, 1972
7) PRAVE, P.; D. SUKATSCH & L. VERTESY: Proticin, a new phosphorus-containing antibiotic. J. Antibiotics 25: 1~10, 1972
8) VÉZINA, C.; A. KUDELSKI & S. N. SEHGAL: Rapamycin (AY-22,989), a new antifungal antibiotic. J. Antibiotics 28: 721~732, 1975
9) GIBBS, B. M. & F. A. SKINNER: Identification methods for microbiologists. Part A. Soc. Appl. Bact., Technical Ser. 1, Academic Press, New York and London, 1966
10) GIBBS, B. M. & D. A. SHAPTON: Identification methods for microbiologists. Part B. Soc. Appl. Bact., Technical Ser. 2, Academic Press, New York and London, 1968
11) IIZUKA, H. & T. SETO: Identification of Bacteria. I, II and III. Hakkokoza, Hakkokyokaisi 20: VII, 14~25, 1962 (In Japanese)
12) BUCHANAN, R. E. & N. E. BIBBONS: BERGEY's Manual of Determinative Bacteriology. 8th edition, The Williams & Wilkins Co., Baltimore, p. 529, 1974
13) SKERMAN, V. B. D.: A guide to the identification of the genera of bacteria. 2nd edition. Williams and Wilkins, Baltimore, Maryland, 1967
14) O'CONNOR, R. T. & L. A. GOLDBLATT: Correlation of ultraviolet and infrared spectra of terpene hydrocarbons. Anal. Chem. 26: 1726~1737, 1954
15) AIZAWA, S.; M. SHIBUYA & S. SHIRATO: Resistaphylin, a new antibiotic. I. Production, isolation and properties. J. Antibiotics 24: 393~396, 1971