THIOTROPOCIN, A NEW SULFUR-CONTAINING 7-MEMBERED-RING ANTIBIOTIC PRODUCED BY A *PSEUDOMONAS* SP.

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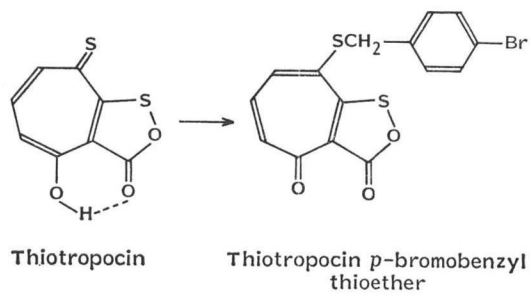
Thiotropocin, a new sulfur-containing 7-membered-ring antibiotic, was isolated from a culture broth of *Pseudomonas* sp. CB-104. The antibiotic occurs as orange or yellowish orange needles and has the molecular formula $C_8H_4O_3S_2$. It is active against Gram-positive and Gram-negative bacteria, some phytopathogens and mycoplasma.

In the course of our screening program for new antibiotics, a bacterium, strain CB-104, identified as a *Pseudomonas* sp., was found to produce a new antibiotic, thiotropocin, whose structure is shown in Fig. 1.[1] This paper describes the taxonomic characterization of strain CB-104, and the fermentation, isolation, physicochemical and biological properties of thiotropocin.

Fig. 1. Structure of thiotropocin.



Thiotropocin — Thiotropocin p-bromobenzyl thioether

Taxonomic Characterization of Strain CB-104

Strain CB-104 was isolated from a soil sample collected in Takarazuka, Hyogo, Japan. The morphological, cultural and physiological properties of this strain are listed in Table 1.

Strain CB-104 is a Gram-negative rod, motile with a polar flagellum, not pleomorphic, not acid-fast, non-spore forming, oxidative, strictly aerobic and catalase-positive; it forms neither fluorescent nor non-fluorescent pigment.

When these characteristics of the strain CB-104 were compared with those of bacteria described in the 8th edition of BERGEY's Manual of Determinative Bacteriology (1974), it was obvious that strain CB-104 is a member of the genus *Pseudomonas* and hence is designated as *Pseudomonas* sp. CB-104. The strain has been deposited in the Institute for Fermentation, Osaka, where it has been assigned the number IFO 14173.

Fermentation

Pseudomonas sp. CB-104 was cultivated at 24°C for 3 days on a slant of nutrient agar (Difco) supplemented with 0.1% of NaCl. One loopful of cells was inoculated into 500 ml of seed medium in a 2-liter Sakaguchi flask. The seed medium contained (g/liter) fructose 10, Polypepton (Daigo Nutritive Chemicals, Ltd.) 5, meat extract (Wako Pure Chemical Ind.) 5, and NaCl 5 (pH 7.0). Two

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Table 1. Characterization of strain CB-104.

Form	Rods	Catalase	+
Size	0.7~1.0×1.5~2.5 μm	Oxygen demand	Aerobic
Mobility	Motile	O-F test	Oxidative
Flagella	Polar, mono	(Hugh and Leifson test)	
Gram stain	Negative	Temperature for growth	4~34°C
Spore	None		(Optimum 18~26)
Reduction of nitrates	+	pH for growth	5~8.5
Denitrification	−		(Optimum 5.5~8.0)
Methyl red test	−	Growth on:	
Voges-Proskauer test	−	Nutrient broth	+
Production of indole	−	Nutrient agar slant	+
Production of hydrogen sulfide	−	Nutrient gelatin stab	+ (No liquefaction)
		Litmus milk	+ (Peptonization)
Hydrolysis of starch	−	Acid production from sugar*	
Utilization of citrate	+	Positive (without gas)	D-Glucose, maltose
Utilization of:		Negative	L-Arabinose, D-xylose, D-mannose, D-fructose, D-galactose, sucrose, lactose, trehalose, D-sorbitol, *i*-inositol, D-mannitol, glycerol, starch
Potassium nitrate	+		
Ammonium sulfate	+		
Production of pigment	−		
Urease	−		
Arginine dihydrorase	−		
Lysine decarboxylase	−		
Oxidase	+ (Weak)		

* Medium: Bacto-peptone (Difco) 1%, sodium chloride 0.5%, bromcresol purple 0.002% (pH 7.5).

such seed flasks were incubated at 28°C for 2 days with reciprocal shaking. The culture broth was then transferred to 120 liters of the seed medium in a 200-liter fermentor and cultivation was carried out at 24°C for one day with aeration (90 liters/minute) and agitation (180 rpm). The culture broth (30 liters) was transferred to 1,200 liters of the main fermentation medium in a 2,000-liter fermentor and cultivation was carried out at 24°C for 42 hours (aeration; 900 liters/minute, agitation; 180 rpm). The main fermentation medium contained (g/liter) fructose 50 (sterilized separately), Polypepton 5, meat extract 5, NaCl 5, and $Na_2S_2O_3$ 0.5 (pH 7.0). A typical fermentation profile is shown in Fig. 2. Thiotoropocin was produced in parallel with bacterial growth and its amount reached a maximum (190 μg/ml) at 36 hours.

Isolation

The filtrate of the culture broth (1,250 liters) was adjusted to pH 3.0 with sulfuric acid and extracted with ethyl acetate. The extracts (460 liters) were washed with water and the active substance was transferred to a 2% $NaHCO_3$ aqueous solution. The transferred solution (220 liters) was acidified to pH 3 and re-extracted with ethyl acetate (110 liters). The extracts were concentrated and crystals of thiotropocin (2.7 g) were obtained. The mother liquor was washed with 0.2% ethylenediaminetetraacetic acid disodium salt solution (5 liters) and subsequently with water, and the ethyl acetate layer was concentrated to give further crystals (7 g).

Physical and Chemical Properties of Thiotropocin

Thiotropocin showed a single spot on thin-layer chromatography (TLC) and a single peak by high-performance liquid chromatography (HPLC) (Table 2). It gave positive reactions with ferric chloride and potassium permanganate reagents, and negative reactions with ninhydrin and Dragendorff reagents.

Fig. 2. Time course of thiotropocin production.
○ Growth, ● thiotropocin, ■ residual sugar.

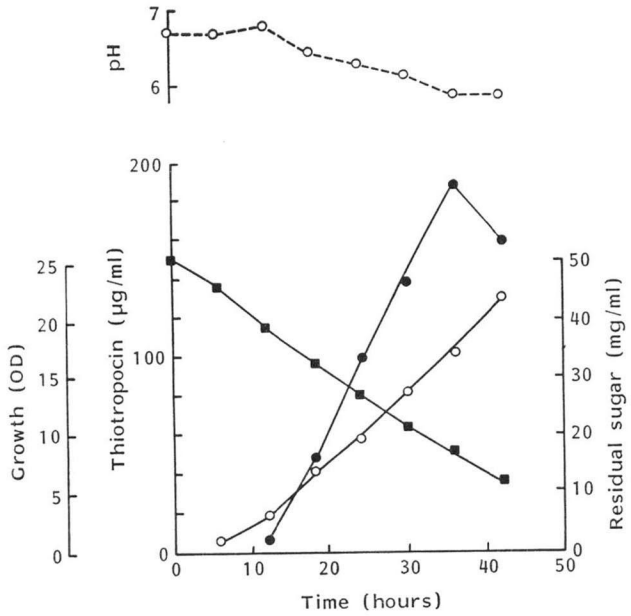
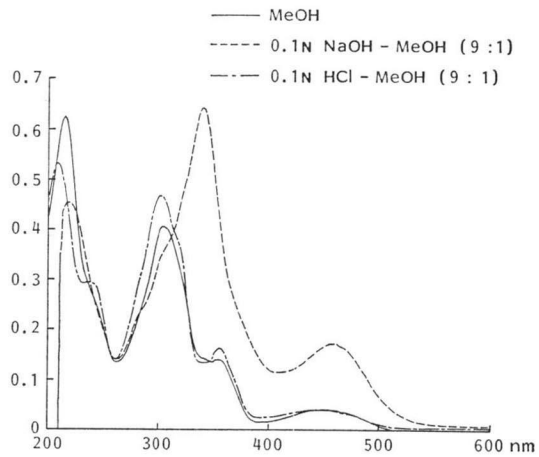


Table 2. Mobility of thiotropocin on TLC and HPLC.

	Solvent system	Rf or Rt (minutes)
TLC*1	$CHCl_3$ - MeOH (4: 1)	0.14
	Toluene - dioxane - CH_3COOH (45: 10: 2)	0.44
	CH_3CN - H_2O (4: 1)	0.48
HPLC*2	MeOH - 0.02 M phosphate buffer (pH 7.6) (1: 4)	6.5

*1 Kiesel gel F_{254} (Merck).
*2 μ-Bondapak C_{18} (3.9 mm × 30 cm, Waters), 2 ml/minute. The eluate was monitored by the absorption at 245 nm.

Fig. 3. UV-visible spectra of thiotropocin.



It was soluble in dimethyl sulfoxide, tetrahydrofuran and chloroform; scarcely soluble in acetone, ethyl acetate and methanol; and insoluble in ethyl ether and water.

Thiotropocin was obtained as orange or yellowish orange needles that began to decompose at 222~225°C but did not show a clear melting point by 300°C. The *pKa'* value was 5.1 by the UV method. The molecular weight was measured by EI-MS as 212 (M^+). Elemental analysis gave the following data: C 45.23, H 1.77, S 30.14 (%). Thus, the molecular formula of thiotropocin was determined to be $C_8H_4O_3S_2$: Calcd. C 45.27, H 1.90, O 22.61, S. 30.22 (%).

The UV-visible spectrum, λ_{max}^{MeOH} (ε) was 216.5 nm (25,000), 245 nm (11,300), 307 nm (16,300),

356 nm (6,200) and 452 nm (2,100). The addition of NaOH shifted the absorption maximum at 307 nm to 343 nm and the subsequent addition of HCl reversed the shift (Fig. 3).

The IR spectrum, ν_{max}^{KBr} (cm^{-1}) was 1630 and 1600. No absorption in the normal carbonyl region was detected (Fig. 4).

The ^1H NMR spectrum of thiotropocin (400 MHz, $CDCl_3$) showed ABX type signals of three hydrogens: δ 7.12 ppm (dd, J=0.20, 9.16 Hz), δ 7.44 ppm (dd, J=0.20, 12.3 Hz), and δ 7.45 ppm (dd, J=9.16, 12.3 Hz), and a singlet signal of one hydrogen (δ 16.7 ppm) that disappeared when D_2O was added (Fig. 5).

The ^{13}C NMR spectrum (100 MHz, DMSO-d_6) showed eight signals, all at regions of carbonyl

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

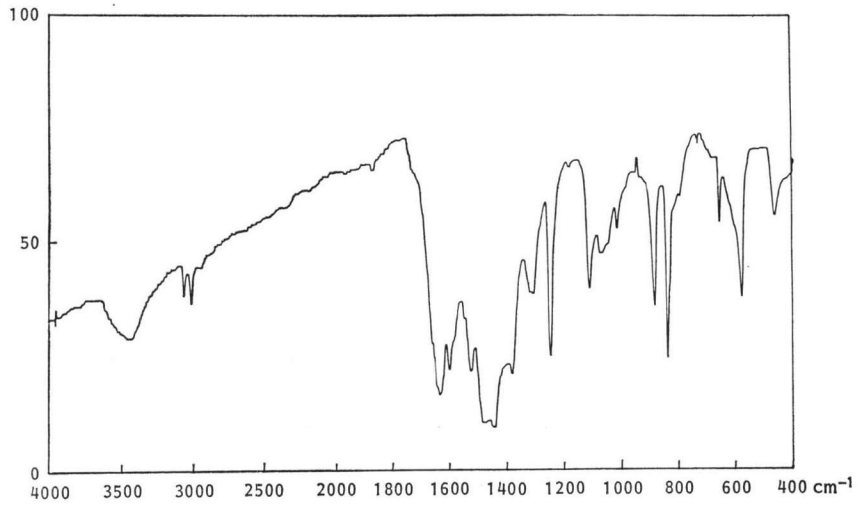
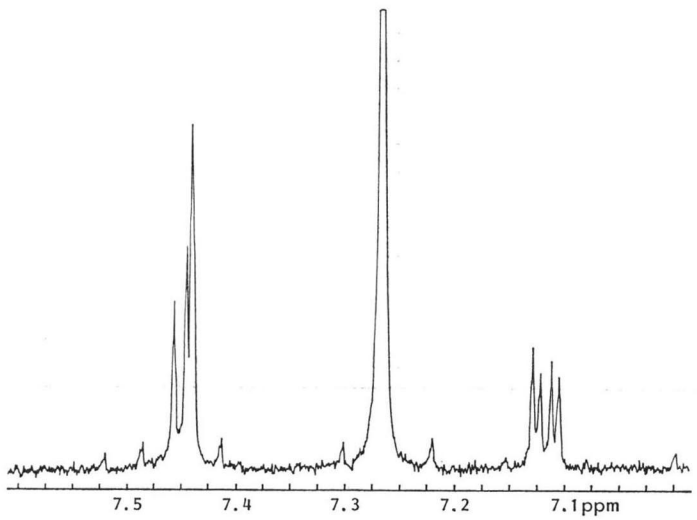


Fig. 5. ^1H NMR spectrum of thiotropocin (400 MHz, $CDCl_3$).

Values in the text were obtained from the analysis of ABX spin system by the use of interactive computer program.



and double bonds: δ ppm 182.62 (s), 170.60 (s), 167.65 (s), 150.09 (s), 137.74 (d), 137.64 (d), 133.80 (d), and 120.04 (s).

The unique structure of thiotropocin was determined by X-ray crystallographic analysis of the *p*-bromobenzyl derivative[1] (Fig. 1).

Biological Properties of Thiotropocin

Thiotropocin exhibited a broad antimicrobial spectrum (Table 3). It was active against Gram-positive and Gram-negative bacteria, fungi and mycoplasma, and showed especially strong activity against some strains of *Proteus*, *Treponema*, *Mycoplasma*, and phytopathogenic fungi. The *in vitro* antimicrobial activity depended on the pH of the assay medium; it was stronger in the acidic range[1] (Table 4).

Thiotropocin caused morphological changes of *Proteus mirabilis* and *Escherichia coli*. In a hypertonic medium, the MIC of thiotropocin caused *P. mirabilis* cells to become spherical (Fig. 6) and *E. coli* cells to swell or become ovoid. In contrast, at a higher concentration (200 μg/ml), no change was observed in *P. mirabilis* and poor lysis in *E. coli*.

The growth of *P. mirabilis* in Penassay broth was immediately inhibited after thiotropocin was added and no drastic decrease of optical density was observed (Fig. 7). This indicates the weak lytic activity of the compound. Immediate growth inhibition was observed whenever thiotropocin was added during the exponential to early stationary phase (Fig. 8).

Table 3. Antimicrobial activity of thiotropocin.

Organism	MIC (μg/ml)	Organism	MIC (μg/ml)
Staphylococcus aureus FDA 209P[a]	3.13	*Treponema hyodysenteriae* DJ 70P1[c]	0.78
Micrococcus luteus IFO 12708[a]	3.13	*T. hyodysenteriae* 78/A[c]	1.56
Bacillus subtilis PCI 219[a]	6.25	*Mycoplasma gallisepticum* S6[d]	12.5
B. cereus FDA 5[a]	12.5	*M. gallisepticum* SAS[d]	0.39
B. sphaericus IFO 12622[a]	12.5	*M. pulmonis* M-53[d]	0.39
Escherichia coli NIHJ JC2[a]	12.5	*Penicillium chrysogenum* IFO 4626[e]	>25
Serratia marcescens IFO 12648[a]	12.5	*Aspergillus niger* IFO 4066[e]	>25
Alcaligenes faecalis IFO 13111[a]	6.52	*A. fumigatus* IFO 6344[e]	25
Proteus mirabilis ATCC 21100[a]	0.78	*Trichophyton mentagrophytes* IFO 7522[e]	25
P. vulgaris IFO 3988[a]	0.78	*Candida albicans* IFO 0583[f]	>25
P. morganii IFO 3168[a]	3.13	*Saccharomyces cerevisiae* IFO 0209[f]	>25
Salmonella typhimurium IFO 12529[a]	12.5	*Alternaria kikuchiana* IFO 7515[g]	6.25
Klebsiella pneumoniae IFO 3317[a]	3.13	*A. mali* IFO 8984[g]	6.25
Citrobacter freundii IFO 12681[a]	12.5	*Cladosporium cucumerinum* IFO 6370[g]	6.25
Comamonas terrigena IFO 12685[a]	12.5	*Helminthosporium sigmoidem* v. *irregulare*[h]	3.13
Acinetobacter calcoaceticus IFO 13006[a]	6.25	*Rizoctonia solani* KHG-2[h]	3.13
Pseudomonas aeruginosa IFO 12689[a]	12.5	*Pyricularia oryzae* IFO 5279[h]	3.13
P. aeruginosa IFO 3080[a]	12.5	*Sclerotinia sclerotiorum* IFO 9395[h]	6.25
Clostridium perfringens PB6K[b]	3.13	*Botryotinia fuckeliana* IFO 7293[i]	25
Bacteroides fragilis ATCC 2509[b]	25		
B. vulgalis ATCC 8482[b]	3.13		

[a] Mueller - Hinton agar (Difco), 37°C, 18 hours. [b] GAM agar (Nissui), Gas Pak (BBL), 37°C, 18 hours. [c] Blood Trypticase Soy Agar (BBL), Gas Pak (BBL), 37°C, 18 hours. [d] Hayflick medium (ref 4), 37°C, 120 hours. [e] 1% Glucose - Trypticase Soy Agar (BBL), 28°C, 40 hours. [f] 1% Glucose - Trypticase Soy Agar (BBL), 28°C, 24 hours. [g] Modified Pfeffer agar (ref 5), 28°C, 40 hours. [h] Same as [g], 28°C, 64 hours. [i] Same as [g], 24°C, 88 hours.

Table 4. Effect of pH on the antibacterial activity of thiotropocin.

Test organism	MIC (μg/ml)*		
	pH 6	pH 7	pH 8
Staphylococcus aureus 209 P	0.39	3.13	50
Bacillus subtilis PCI 219	0.78	6.25	25
Micrococcus luteus IFO 12708	0.20	3.13	25
Escherichia coli NIHJ JC2	1.56	12.5	50
Klebsiella pneumoniae IFO 3317	1.56	3.13	25
Citrobacter freundii IFO 12681	3.13	12.5	50
Proteus mirabilis ATCC 21100	0.20	0.78	12.5
P. vulgaris IFO 3988	<0.1	0.78	12.5
P. morganii IFO 3168	0.39	3.13	25
Pseudomonas aeruginosa IFO 12689	3.13	12.5	>50
Serratia marcescens IFO 12648	3.13	12.5	50

* Mueller-Hinton agar (Difco), 37°C, 18 hours.

Fig. 6. Morphological change of *Proteus mirabilis* ATCC 21100 cells in a hypertonic solution containing thiotropocin.

The morphological change was determined after *P. mirabilis* ATCC 21100 was incubated in Bacto-Penassay Broth containing 20% sucrose and thiotropocin at 37°C for 2 hours with shaking.

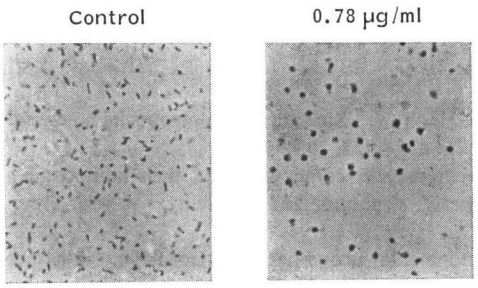


Fig. 8. The effect of thiotropocin (0.78 μg/ml) on the growth of *P. mirabilis* ATCC 21100.

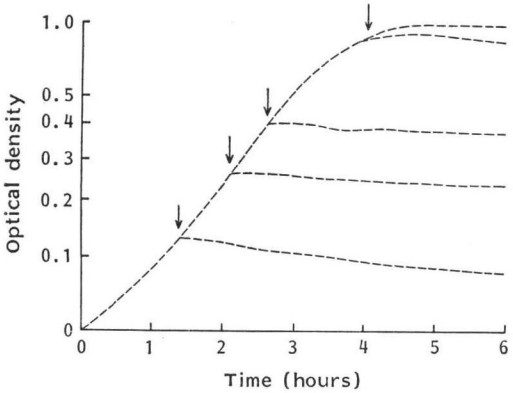
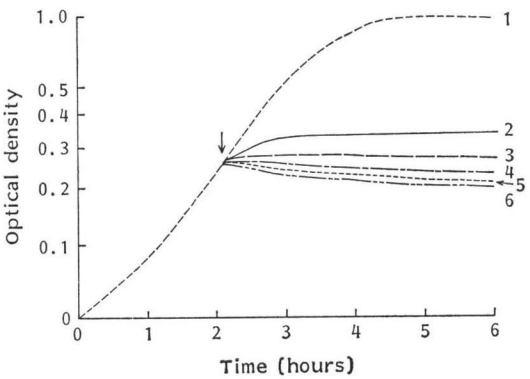


Fig. 7. The effect of thiotropocin on the growth of *P. mirabilis* ATCC 21100.

The cultures were incubated at 37°C in Bacto-Penassay Broth (Difco Laboratories) with shaking; various concentrations of thiotropocin were added at the exponential phase and changes in turbidity were monitored with the biophotometer BIO-Log II (Jasco Jouan).

1: Control, 2: 6.25 μg/ml, 3: 3.13 μg/ml, 4: 1.56 μg/ml, 5: 0.78 μg/ml, 6: 0.39 μg/ml.



The acute toxicity (LD_{50}) of the antibiotic in mice was about 50~100 mg/kg by oral administration and about 8 mg/kg by subcutaneous injection.

Discussion

Several 7-membered-ring aromatic tropolone compounds have been reported. As fungal metabolites, stipitatic and stipitatonic acids were isolated from *Penicillium stipitatum*; puberulic and puberulonic acids from *P. puberulum* and several species of *Penicillium*; and sepedonin from *Sepedonium chrysospermum*. Hinokitiol (β-thujaplicin) α-thujaplicin, and γ-thujaplicin were isolated as plant

constituents,[2] and colchicine and related compounds as alkaloids. Recently, tropothione was chemically synthesized and its physico-chemical properties characterized; it was found to be very unstable.[3]

Thiotropocin, a new tropothione antibiotic was discovered as a bacterial metabolite. Thiotropocin is the first example of a naturally occurring tropothione derivative and has a unique structure containing an -S-O-CO- moiety.

TRUST *et al.* reported on the antibacterial activity of β-thujaplicin[6] and tropolone.[7] The MICs (μg/ml) of β-thujaplicin against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* were 31, 62.5, and 31, respectively. The MICs of tropolone were found to be of the same order. On the other hand, thiotropocin showed strong antibacterial activity. The MICs of thiotropocin against these microorganisms were 6.25, 3.13, and 12.5, respectively. β-Thujaplicin and tropolone have weak antibacterial activity and comparatively strong lytic activity.[6,7] The results obtained with thiotropocin suggest that its mode of action is different from those of tropolone derivatives.

Recently, 7-hydroxytropolone was isolated as a fermentation product of *Pseudomonas*[8] and *Streptomyces neyagawaensis*,[9] independently, and found to possess a specific aminoglycoside-2″-*O*-adenyltransferase inhibitory activity.[9,10]

Studies of the mode of action, biosynthesis and chemistry of thiotropocin may have a unique impact on the field of naturally occurring 7-membered ring compounds.

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References

1) TSUBOTANI, S.; Y. WADA, K. KAMIYA, H. OKAZAKI & S. HARADA: Structure of thiotropocin, a new sulfur-containing 7-membered antibiotic. Tetrahedron Lett. 25: 419～422, 1984
2) PIETRA, F.: Seven-membered conjugated carbo- and heterocyclic compounds and their homoconjugated analogs and metal complexes. Synthesis, biosynthesis, structure, and reactivity. Chem. Rev. 73: 293～364, 1973
3) MACHIGUCHI, T. & H. OTANI: The isolation and fundamental properties of tropothione. Abstracts of 15th Symposium on Nonbenzenoid Aromatic Compounds, pp. 244～247, 1982
4) CHANOCK, R. M.; L. HAYFLICK & M. F. BARILE: Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a pleuropneumonialike organism. Proc. Nat. Acad. Sci., U.S.A. 48: 41～48, 1962
5) IWASA, T.; E. HIGASHIDE & M. SHIBATA: Studies on validamycins, new antibiotics. III. Bioassay methods for the determination of validamycin. J. Antibiotics 24: 114～118, 1971
6) TRUST, T. J. & R. W. COOMBS: Antibacterial activity of β-thujaplicin. Can. J. Microbiol. 19: 1341～1346, 1973
7) TRUST, T. J.: Antibacterial activity of tropolone. Antimicrob. Agents Chemother. 7: 500～506, 1975
8) KORTH, H.; G. PULVERER, A. ROME & H. BUDZIKIEWICZ: 7-Hydroxytropolon aus *Pseudomonas* sp. Z. Naturforsch. 36C: 728～729, 1981
9) ALLEN, N. E.; W. E. ALBORN, Jr., J. N. HOBBS, Jr. & H. A. KIRST: 7-Hydroxytropolone: An inhibitor of aminoglycoside-2″-*O*-adenyltransferase. Antimicrob. Agents Chemother. 22: 824～831, 1982
10) KIRST, H. A.; G. G. MARCONI, F. T. COUNTER, P. W. ENSMINGER, N. D. JONES, M. O. CHANEY, J. E. TOTH & N. E. ALLEN: Synthesis and characterization of a novel inhibitor of an aminoglycoside-inactivating enzyme. J. Antibiotics 35: 1651～1657, 1982