ACTINOTIOCIN, A NEW SULFUR-CONTAINING PEPTIDE ANTIBIOTIC FROM *ACTINOMADURA PUSILLA*

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(Received for publication March 28, 1973)

Actinotiocin is a new sulfur-containig peptide antibiotic obtained from the cultured broth and the mycelium of *Actinomadura pusilla* A-118 (S_2-118). It is extracted with ethyl acetate and purified by silica gel chromatography. It forms colorless columnar crystals, melts at 247~249°C, and gives $[\alpha]_D^{20}+164°$ (*c* 0.77, dioxane). $C_{49}H_{53-55}N_{13}O_{10}S_5$ was suggested for its molecular formula by elemental analysis. On acidic hydrolysis of actinotiocin, a new large molecular amino acid (**III**, $C_{28}H_{18}N_6O_6S_4$) having some heteroaromatic rings, serine, proline, glycine and unidentified amino acids were obtained. The partial structure of the amino acid (**III**) was deduced from the chemical reaction and spectral data. Actinotiocin showed strong antibacterial activity against gram-positive bacteria and no cross resistance with commercial antibiotics. The administration of 1,000 mg/kg of this antibiotic into mice by intraperitoneal and oral routes did not result in any toxic symptoms.

In a continuing search for less known genera of the Actinomycetales as producers of new antibiotics, *Actinomadura pusilla* A-118 (S_2-118) was found to produce a new antibiotic with activity against staphylococci resistant to various antibiotics. Chemical studies showed that this antibiotic is a new sulfur-containing peptide antibiotic, and it has been named actinotiocin.

Actinomadura pusilla A-118 (S_2-118) was isolated from a soil sample collected in Kofu City and described by NONOMURA and OHARA in 1971[1]. It was found to produce an *in vitro* activity against *Staphylococcus aureus*.

We received strain S_2-118 from Dr. NONOMURA and investigated the fermentation condition ecessary for the production and the isolation of the antibiotic. In this paper, we describe the production, isolation and some characteristics of actinotiocin.

Production of Antibiotic

The culture of strain S_2-118 was maintained on yeast extract-malt extract agar (ISP medium 2)[2]. Fermentation conditions suitable for the production were studied and the following medium was found to be useful. Vegetative and fermentation medium (g/liter): maltose, 20; meat extract, 15; pH 7.0 (adjusted to 7.0 prior to sterilization).

For the production of the antibiotic, a 100-liter fermentorco ntaining 50 liters of the medium was inoculated with 3.5 liters of vegetative culture and incubated aerobically (1 liter air/liter/min.) under stirring (250 r.p.m.) at 38°C.

Maximum antibiotic activity was obtained after 72 hours of fermentation. A paper disk agar plate assay with *Staphylococcus aureus* TERAJIMA as a test organism was used to determine the antibiotic levels.

Isolation of Actinotiocin

Actinotiocin was isolated from both the broth and mycelia by organic solvent extraction procedures. The cultured broth (50 liters, pH 8.4) was separated continuously in S-type ultracentrifuge at 10,000 r.p.m.

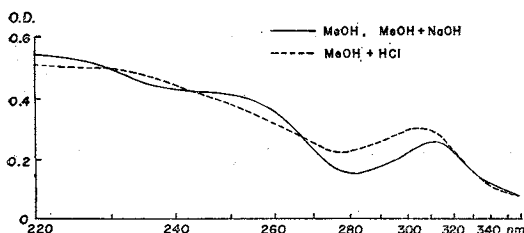
The wet mycelial cake (2.3 kg) was extracted two times with 5-liter portion of 80 % aqueous acetone. The extracts were combined and concentrated *in vacuo* to an aqueous solution (3 liters). The aqueous solution was extracted six times with 2-liter portion of ethyl acetate. The ethyl acetate extracts were combined and concentrated *in vacuo* to dryness and dissolved in 40 ml of ethanol-chloroform (1 : 1, v/v) and precipitated by adding 300 ml of *n*-hexane. A crude substance of actinotiocin was obtained (1.8 g). The crude actinotiocin was purified by chromatography on a column of silica gel. The crude substance was dissolved in 200 ml of methanol-chloroform (1 : 50, v/v) and charged on top a column to which a suspension of 60 g silica gel (Mallinckrodt, 100 mesh) in chloroform had previously been applied. The chromatographic development was carried out by stepwise elution with 750 ml of 2 % (v/v) methanol-chloroform, 1,500 ml of 3 % (v/v) methanol-chloroform and 1,500 ml of 4 % (v/v) methanol-chloroform, and actinotiocin was eluted late with 3 % methanol-chloroform. The antibacterial activity of the elutes was monitored by silica gel TLC and bioautogram. The active fractions were combined and concentrated *in vacuo* to dryness (1.1 g). By the addition of 35 ml hot methanol and standing at room temperature, actinotiocin was precipitated to colorless columnar crystals (700 mg), and purified by recrystallization from hot methanol (648 mg).

In the case of the supernatant broth, the broth (50 liters) was extracted two times with 25-liter portion of ethyl acetate. By the same procedures above, the crude substance, crystals and recrystals of actinotiocin from the ethyl acetate extracts were 890 mg, 670 mg and 579 mg successively.

Physico-Chemical Characteristics

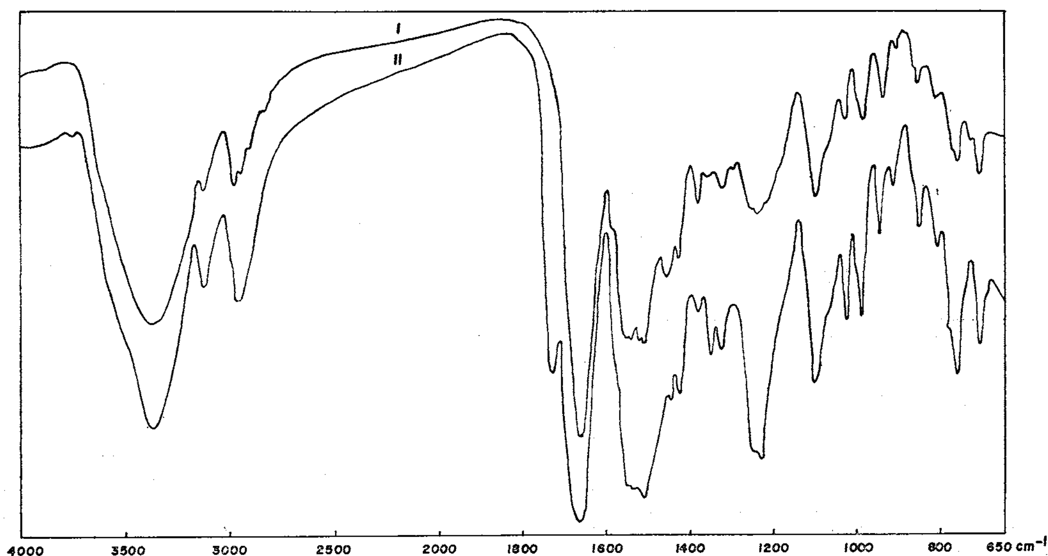
Actinotiocin (I), colorless columns, m.p. 247~249°C, $[\alpha]_D^{20}+164°$ (c 0.77, dioxane), is soluble in methanol-chloroform mixture, ethanol-chloroform mixture, DMSO and dioxane, slightly soluble in methanol, ethanol, acetone, chloroform and ethyl acetate, but insoluble in water, *n*-hexane and benzene. Its UV spectra are shown in Fig. 1: λ_{max}^{MeOH} 310~311 nm log ϵ 4.47 ($E_{1cm}^{1\%}$ 256), 340 nm (sh) log ϵ 4.14, $\lambda_{max}^{NaOH-MeOH}$ 310~311 nm, $\lambda_{max}^{HCl-MeOH}$ 304 nm. The IR spectrum taken in KBr disk is shown in Fig. 2. The NMR spectrum taken in DMSO-d_6 solution (Varian A-60) is shown in Fig. 3. Elementary analysis, measurement of the molecular weight (1,507) by the vapor pressure equilibrium method (Hitachi Perkin-Elemer Model 115, $CHCl_3$-MeOH (1 : 1) solution) and the number of protons in the NMR spectrum proposed a molecular formula of $C_{49}H_{53-55}N_{13}O_{10}S_5$ (MW 1,144~1,146) for **I**. Analysis Calcd. for $C_{49}H_{53}N_{13}O_{10}S_5$: C, 51.43; H, 4.67; N, 15.92; S, 14.01 (%). Analysis Calcd. for $C_{49}H_{55}N_{13}O_{10}S_5$: C, 51.34; H, 4.84; N, 15.89; S, 13.98 (%). Analysis Found: C, 51.66, 51.66; H, 4.52, 4.30; N, 15.92, 16.16; S, 13.94, 14.08 (%).

Fig. 1. UV spectra of actinotiocin



On thin-layer chromatography using silica gel, Rf value of **I** is 0.39 with MeOH-$CHCl_3$ (1:9), and the spot is visualized under UV lamp.

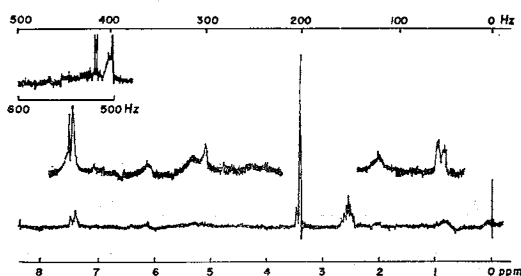
Fig. 2. IR spectra of actinotiocin (I) and deamino-actinotiocin (II) (KBr disk).



The spot of I gives negative ninhydrin test.

Actinotiocin (I) was stable in neutral and alkaline condition, but was so unstable in acidic condition that ammonia and deaminoactinotiocin hydrochloride (II) were produced on mild hydrolysis with 0.01 N HCl. The IR spectrum of II (Fig. 2) is similar to that of I except a band at 1730 cm^{-1}. Actinotiocin was hydrolyzed with 6 N HCl at 115°C for 24 hours in a sealed tube. The hydrolysate contained a crystalline compound III and at least five ninhydrin-positive products.

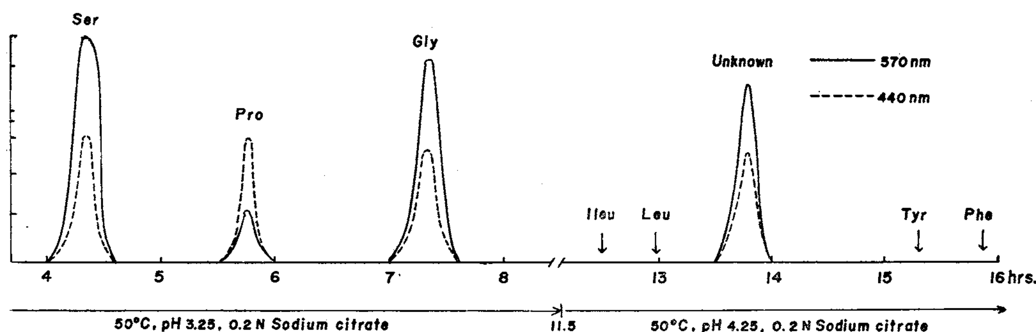
Fig. 3. NMR spectrum of actinotiocin in DMSO-d_6 solution.



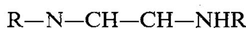
Compound III, colorless needles, m.p. 220~225°C (dec.), $[\alpha]_D^{29}+57.8°$, $C_{28}H_{18}N_6O_6S_4 \cdot HCl$, was ninhydrin-negative and showed a carbonyl absorption at 1700 cm^{-1} in its IR spectrum. Its UV spectral data (λ_{max}^{EtOH} 308 nm log ϵ 4.47, 304 nm (sh) log ϵ 4.17) indicated that III had the main part of a chromophore presented in I. The NMR spectrum taken in DMSO-d_6 solution exhibited two methine protons at δ 5.47 and 5.15, and the following ten aromatic protons; δ 7.39 (4 H, s: singlet), 7.98 (1 H, s), 8.33 (1 H, s), 8.60 (1 H, s), 8.69 (1 H, s), 8.45 (1 H, d: doublet, J=8.5 Hz) and 8.57 (1 H, d, J=8.5 Hz). Methylation of III with methanolic hydrochloric acid gave a dimethylester hydrochloride (IV-HCl). Absorption of IV-HCl at 1715 cm^{-1} in the IR spectrum and two singlets at δ 3.92 and 3.63 in the NMR spectrum of IV-HCl were attributed to two carbomethoxyl groups. On acetylation with acetic anhydride-pyridine, IV afforded a di-N-acetyldimethylester (V). The IR spectrum of V exhibited ester and amide carbonyl bands at 1730 and 1655 cm^{-1}, respectively. Further, its NMR spectrum revealed the presence of two carbomethoxyl groups at δ 3.97 and 4.02, and two acetyl groups at δ 2.00 and 2.10.

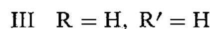
On the NMR spectral analysis, a pair of multiplet signals of vicinal methine proton was recognized in III, IV-HCl and IV at δ 5.15 and 5.47, δ 5.15 and 5.49, and δ 4.47 and 4.89, respective-

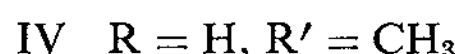
Fig. 4. Chromatography of amino acids of hydrolyzed actinotiocin (Amberlite CG-120 Type III)



ly. After addition of deuterium oxide, these pairs of vicinal methine proton collapsed to clean AB type quartet (J=3.5 Hz in **III**, 4.5 in **IV**-HCl, and 7.0 in **IV**). Furthermore, the NMR spectrum of **V** exhibited a doublet NH-proton at δ 6.56 (J=8.5 Hz), a doublet methine proton at δ 6.37 (J=5.8 Hz), and a double-doublet methine proton at δ 5.88 (J=5.8 and 8.5 Hz). The double-doublet signal at δ 5.88 was collapsed to a clean doublet (J=5.8 Hz) by deuterium exchange of NH-proton. These NMR spectral data suggested the presence of the tentative partial structure A in **III**.







A

The UV spectral data of **II** is analogous to that of 2-(2-aminoethyl)-2, 4-bithiazole-4-carboxylic acid[8]. Further, the signals of aromatic protons in the NMR spectrum of **III** appear at low field and some of them are sharp singlets. From these facts, it may be considered that the rest of compound **III** comprises some heteroaromatic rings containing N and S atoms, *e.g.* thiazole. Therefore, compound **III** seems to be a new large-molecule amino acid.

On the other hand, serine, proline, glycine and an unknown amino acid with ratio 1 : 1 : 1 : 1 were detected in the hydrolysate of actinotiocin by means of chromatography on Amberlite CG-120 Type ion-exchange resin, as shown in Fig. 4. The paper chromatography and high voltage paper electrophoresis of the hydrolysate gave five ninhydrin-positive spots in which serine, proline and glycine were identified by the comparison with authentic samples. But no further identification of unknown amino acids was made.

Partial Hydrolysis of Actinotiocin (I)

(1) To a solution of **I** (0.2 g) in a mixture of MeOH (50 ml) and $CHCl_3$ (25 ml), 0.01 N HCl (5 ml) was added. The reaction mixture was allowed to stand at room temperature for 12 hours. After evaporation of solvents, the residue was purified on a silica gel column to give deamino-actinotiocin (**II**), colorless powders, m. p. 233~236°C, $[\alpha]^{25}_{D}$+110.9° (*c* 1.29, dioxane). Anal. Calcd. for $C_{49}H_{50}N_{12}O_{10}S_5 \cdot 2HCl$: C, 49.03; H, 4.37; N, 14.01; S, 13.36; Cl, 5.91. Calcd. for $C_{49}H_{52}N_{12}O_{10}S_5 \cdot 2HCl$: C, 48.94; H, 4.53; N, 13.98; S, 13.33; Cl, 5.90. Found: C, 49.03; H, 4.18; N, 14.12; S. 13.14; Cl, 6.30. Rf 0.91 (on Eastman Chromagram sheet with $CHCl_3$-MeOH (9:1)).

(2) To a solution of **I** (2 mg) in a mixture of MeOH (10 ml) and $CHCl_3$ (5 ml), 0.01 N HCl (0.05 ml) was added. After standing for 12 hours, the reaction mixture was analyzed by auto amino acid analyser to detect 24.1 mcg (80 %) of ammonia.

Compound **III**

A mixture of **I** (0.98 g) and 6 N HCl (39 ml) was heated at 115°C for 24 hours in a sealed tube. After cooling, the separated crystals were collected and recrystallized from acetone-1 N HCl to give colorless needles (**III**) (0.5 g), m.p. 220~225°C (dec), $[\alpha]^{29}_{D}+57.8°$ (*c* 0.74, DMSO). Anal. Calcd. for $C_{28}H_{18}N_6O_6S_4 \cdot HCl$: C. 48.10; H, 2.74; N, 12.02; S, 18.34; Cl, 5.07. Found: C, 47.76; H, 3.08; N, 11.90; S, 17.50; Cl, 4.90. The mother liquor of **III** was used for the analysis of other amino acids as described in the text.

Dimethylester (IV) of Compound **III**

A mixture of **III** (0.25 g) and 10 % HCl-MeOH (20 ml) was refluxed for 40 minutes. The separated crystals were colorless to give collorless needles (**IV**-HCl) (0.23 g), m.p. 215~217°C. UV: λ_{max}^{EtOH} 306 nm log ε 4.52, 340 nm (sh) log ε 4.16. NMR (δ in DMSO-d_6): aromatic protons; 8.76 (1 H, s), 8.67 (1 H, s), 8.54 (1 H, d, J=8 Hz), 8.43 (1 H, d, J=8 Hz), 8.32 (1H, s), 7.97 (1 H, s) and 7.38 (4 H, s), methine protons; 5.49 (1 H, m: multiplet) and 5.15 (1 H, m), $COOCH_3$; 3.92 (3 H, s) and 3.67 (3 H, s). Anal. Calcd. for $C_{30}H_{22}N_6O_6S_4 \cdot HCl$: C, 49.54; H, 3.19; N, 11.56; S, 17.63; Cl, 4.88. Found: C, 49.26; H, 3.31; N, 11.70; S, 17.01; Cl, 5.08. Free base (**IV**), colorless needles m.p. 128~130°C (CH_2Cl_2-EtOH). UV: λ_{max}^{EtOH} 306 nm log ε 4.51, 340 nm (sh) log ε 4.16. NMR

Table 1. *In vitro* antimicrobial activity of actinotiocin. (Serial dilution method)

Test organisms	Medium	MIC(mcg/ml)
Staphylococcus aureus Terajima	(1)	0.03
S. aureus 209 P JC-1	(1)	0.03
S. aureus ATCC 6538	(1)	0.03
Diplococcus pneumoniae I	(2)	0.1
D. pneumoniae II	(2)	0.3
D. pneumoniae III	(2)	0.3
Streptococcus hemolyticus A 65	(2)	0.3
Bacillus subtilis PCI 219	(1)	0.1
Listeria monocytogenes LI-2402	(1)	0.01
Escherichia coli K-12	(1)	>1,000
Shigella flexneri 2 a EW 10	(1)	1,000
S. sonnei EW 33	(1)	>30
Salmonella typhimurium S-9	(1)	>30
Proteus vulgaris OX 19	(1)	>30
Klebsiella pneumoniae No. 13	(1)	>30
Pseudomonas aeruginosa Tsuchijima	(1)	>30
Mycoplasma gallisepticum S-6	(6)	0.3
M. gallisepticum KP-13	(6)	0.1
M. gallisepticum IRF	(6)	0.3
Haemophilus gallinarum 227	(7)	>100
H. gallinarum 260 (SM-resistant)	(7)	>100
Mycobacterium tuberculosis $H_{37}Rv$	(3)	1
M. tuberculosis kurono	(3)	0.3
M. tuberculosis $H_{37}Rv$ (INH, PAS, SM-resistant)	(3)	0.3
Candida albicans ATCC 10257	(4)	>30
Trichophyton mentagrophytes	(4)	>30
Trichomonas vaginalis 4 F	(5)	>30

(1) Nutrient broth, pH 7.2
(2) Brain heart infusion broth, pH 7.4
(3) 0.2 % Bovine albumin-KIRCHNER, pH 7.0
(4) 4 % Glucose-SABOURAUD, pH 5.6
(5) Serum-yeast extract semisynthetic medium
(6) Chick PPLO broth
(7) Chick meat bouillon

(δ in $CDCl_3$): aromatic protons; 8.36 (4H, m), 8.06 (1 H, s), 7.49 (1 H, s), and 7.35 (4 H, s), methine protons; 4.89 (1 H, m) and 4.47 (1H, m), $COOCH_3$; 4.01 (3 H, s) and 3.96 (3 H, s). Anal. Calcd. for $C_{30}H_{22}N_6O_6S_4$: C, 52.16; H, 3.21; N, 12.17; S, 18.56. Found: C, 52.11; H, 3.40; N, 12.40; S, 18.47.

Diacetate (V) of Compound **IV**

Compound **IV** (138 mg) was acetylated by pyridine (2 ml) and Ac_2O (1 ml) at room temperature. The reaction mixture was worked up in the usual manner to give colorless needles (V), m. p. 262~264°C (CH_2Cl_2-MeOH). UV: λ_{max}^{EtOH} 306 nm log ϵ 4.51, 340 nm (sh) log ϵ 4.15. NMR (δ in $CDCl_3$): aromatic protons; 8.38 (3 H, s), 8.35 (1 H, s), 8.10 (1 H, s), 7.47 (1 H, s) and 7.35 (4 H, s), NH; 6.56 (1 H, d, J=8.5 Hz), methine protons; 6.37 (1 H, d, J=5.8 Hz) and 5.88 (1 H, dd, J=5.8 and 8.5 Hz), $COCH_3$; 4.02 (3 H, s) and 3.97 (3H, s), $COOCH_3$; 2.10 (3 H, s) and 2.00 (3 H, s). Anal. Calcd. for $C_{34}H_{26}N_6O_8S_4$: C, 52.70; H, 3.38; N, 10.85; S, 16.55; mol. wt. 774. Found: C, 53.09; H, 3.46; N, 11.03; S, 16.40; mol. wt. 760 (vapor pressure equilibrium osmometry, $CHCl_3$-MeOH (1 : 1) solution).

Biological Characteristics

The antimicrobial activity of actinotiocin is summarized in Tables 1 and 2. Actinotiocin was effective against gram-positive bacteria, *Mycobacteria* and *Mycoplasma*. However, actinotiocin was not effective against gram-negative bacteria, *Candida*, *Trichophyton* and *Trichomonas*. The administration of 1,000 mg/kg of this antibiotic into mice by intraperitoneal and oral routes did not

Table,2. *In vitro* antibacterial activity of actinotiocin against resistant staphylococci (Streaking method). medium: Heart infusion agar, pH 7.4.

Test organisms	MIC (mcg/ml)	Resistance
Staphylococcus aureus Terajima	0.1	
S. aureus Miyamoto	0.03	PC
S. aureus FDA 209 P SM, STH-r	0.1	SM, KM, STH
S. aureus KM, STH-r	0.03	PC, EM, CP, TC, KM, STH
S. aureus SM, STH-r	0.03	SM, KM, STH
S, aureus S-21	0.1	EM
S. aureus P-7c	0.3	PC, TC, SM
S. aureus P-32	0.3	TC, SM
S. aureus P-213	0.3	PC, SM
S. epidermidis No. 8	0.1	PC, CP, SM

PC: Penicillin EM: Erythromycin CP: Chloramphenicol
TC: Tetracycline SM: Streptomycin KM: Kanamycin
STH: Streptothricin

Table 3. Acute toxicity of actinotiocin in mice
Mice: dd/Y-F female mice (23~25 g) Dosage form: suspended in 0.2 % CMC
Term of observatson: 7 days

Route	Dosage (mg/kg)	Dead/tested	Body weight (g)		LD_{50} (mg/kg)
			initial	terminal	
ip	1,000	0/3	24.5*	25.1*	
	500	0/3	23.9	26.5	1,000
	250	0/3	23.4	25.1	
po	1,000	0/3	24.2	25.3	1,000
	500	0/3	23.7	25.7	

* average of 3 mice

result in any toxic symptoms (Table 3).

Against the intraperitoneal infection with *S. aureus* 10 and *D. pneumoniae* I in mice, actinotiocin (100 mg/kg/dose) was effective (70 % and 100 % protection, respectively, in 10 mice after 7 days) by intraperitoneal administration, but ineffective when given orally. Against the intravenous infection with *S. aureus* No. 50774, actinotiocin was not effective by intraperitoneal administration (100 mg/kg/dose). The plasma level (<0.3 μg/ml) and urine level (<0.3 μg/ml) of actinotiocin were not detected by bioassay in five mice given 100 mg/kg orally.

Discussion

On the basis of the investigation described above, actinotiocin is a member of sulfur-containing peptide antibiotics, and related to known antibiotics such as multhiomycin[4], thiopeptins[5,6], siomycins[7], pepthiomycins[8], thiostrepton[9], A-59[10], sporangiomycin[11], thermothiocin[12] and sulfomycins[13].

From the viewpoint of elemental analysis, melting point, optical rotation, IR and UV spectra and degradation products, it seems reasonable to conclude that actinotiocin is a new antibiotic.

Actinotiocin is strongly active against gram-positive bacteria *in vitro*, but not active against gram-negative bacteria and fungi.

From viewpoints of the *in vitro* and *in vivo* antibacterial activity and the acute toxicity, the biological properties of actinotiocin seemed to related to the previously reported sulfur-containing peptide antibiotics.

Acknowledgements

The authors express deep thanks to Dr. H. NONOMURA, Faculty of Engineering, Yamanashi University, for the supply of strain S_2-118 and his helpful advices, and to Director Dr. H. TAKAMATSU, Dr. Y. YOSHIMURA, Dr. H. NISHIMURA and Dr. M. SHIMIZU, Research Laboratories, Dainippon Pharmaceutical Co., Ltd., for their encouragement throughout this work. Thanks are also due to the members of Analytical Center and Chemotherapy Laboratory of our Research Laboratories for microanalyses, spectroscopic measurements, amino acids analysis, MIC and acute toxicity testings respectively.

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