ISOLATION AND CHARACTERIZATION OF THIOXAMYCIN

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A new peptide antibiotic named thioxamycin which contained thiazole and oxazole rings was isolated from the culture broth of *Streptomyces* sp. The antibiotic is acidic and lipophilic in nature. A molecular formula, $C_{52}H_{48}N_{16}O_{15}S_4$, was indicated by elemental analysis and MS. One mol of threonine and three unusual amino acids were detected by amino acid analysis of the acid hydrolysate. The antibiotic is active *in vitro* against anaerobic Gram-positive and Gram-negative bacteria and also aerobic Gram-positive bacteria.

In the course of our screening program for new antibiotics active against anaerobic bacteria, we noticed an antibiotic produced by *Streptomyces* sp. strain PA-46025. Isolation and characterization revealed the antibiotic to be an acidic and lipophilic peptide containing a considerably high content of sulfur and exhibiting an absorption at 252 nm. Detailed comparison with known antibiotics clarified that the antibiotic is most closely related to sulfomycins I and II[1,2], but differentiated from this group by its acidic nature and antibacterial activity. Thioxamycin differs notably from the sulfomycin in its elemental sulfur content despite the fact that the structure of this antibiotic (Fig. 1), which will be discussed in an accompanying paper[3], contains thiazole and oxazole ring moieties as does that of sulfomycins I and II and berninamycin A[2]. The name, thioxamycin, is given to this antibiotic.

In this paper, the taxonomy of the producing strain, the production and isolation of the antibiotic as well as physico-chemical and biological properties of the antibiotic are presented.

Fig. 1. Structure of thioxamycin.

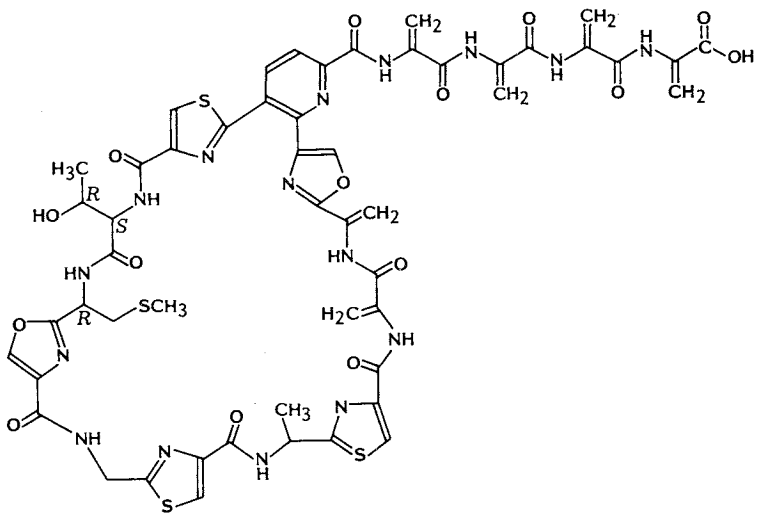


Table 1. Cultural characteristics of strain PA-46025.

Media	Growth	Sporulation	Color of aerial mycelium	Reverse color	Soluble pigment
Sucrose-nitrate agar	Good	Poor	Light brownish gray	Pale yellowish brown	None
Glucose-asparagine agar	Good	Moderate	Light brownish gray	Pale yellowish brown	None
Glycerol-asparagine agar	Good	Good	Light brownish gray	Dull yellow	Dull yellow
Inorganic salts-starch agar	Good	Good	Grayish brown (hygroscopic)	Pale yellowish brown	None
Tyrosine agar	Good	Good	Light brownish gray	Brown	Yellowish brown
Nutrient agar	Good	None	—	Pale yellowish brown	None
Yeast extract-malt extract agar	Good	Good	Brownish gray (hygroscopic)	Dark yellowish brown	Yellowish brown
Oatmeal agar	Good	Good	Grayish brown	Yellowish brown	None
BENNETT's agar	Good	Good	Brownish gray (hygroscopic)	Dark yellowish brown	Yellowish brown

Taxonomy of the Producing Strain

Morphology

The vegetative mycelia of the strain PA-46025 grow well on both synthetic and organic media, and do not show fragmentation into coccoid or bacillary elements. Abundant aerial mycelia are formed on yeast extract-malt extract agar and inorganic salts-starch agar. The spore chains are of the spirales type and have more than twenty spores per chain. The spores are cylindrical in shape, and have smooth surface. Sporangia, sclerotia and flagellated spores were not observed.

Table 2. Physiological properties of strain PA-46025.

Production of melanoid pigment	Positive
Tyrosinase reaction	Negative
Coagulation of milk	Negative
Peptonization of milk	Positive
Hydrolysis of starch	Positive
Liquefaction of gelatin	Positive

Chemical Composition

LL-2,4-Diaminopimelic acid (A_2pm) was detected in the whole-cell hydrolysates of the strain PA-46025 by the method of BECKER *et al.*[4].

Cultural and Physiological Characteristics

Cultures were observed after incubation at 28°C for 2 weeks. On some media, such as yeast extract-malt extract agar, inorganic salts-starch agar, moist black (hygroscopic) areas were observed. The cultural and physiological characteristics of the strain PA-46025 were summarized in Tables 1 and 2, respectively. Color names described in Table 1 were designated on the basis of the color table in "Guide to Color Standard", published by Nippon Shikisai Kenkyusho, Tokyo, Japan.

Carbon Utilization

The utilization of carbon sources was studied on PRIDHAM and GOTTLIEB's basal agar containing 1% of each carbon source incubated at 28°C. D-Glucose, D-xylose, D-fructose, sucrose, inositol, raffinose and D-mannitol were utilized; L-arabinose and L-rhamnose were not utilized.

Strain PA-46025 exhibits the following properties; spore chain, spirales; spores, cylindrical and smooth surface; color of vegetative mycelia, pale yellowish brown to yellowish brown; color of aerial mycelia, light brownish gray to brownish gray; soluble pigment, none or yellowish brown; A_2pm in whole-cell hydrolysates, LL-type.

Based on the taxonomic properties described above, the strain PA-46025 is considered to belong to

the genus *Streptomyces*.

Fermentation

The spores of the strain PA-46025 were inoculated to a 3-liter Erlenmeyer flask containing 800 ml of a medium consisting of soluble starch 0.5%, glucose 0.5%, Polypeptone 0.5%, beef extract 0.5%, yeast extract 0.25%, NaCl 0.25% (pH 7.0). It was cultured at 28°C for 2 days on a rotatory shaker (180 rpm). Four hundred ml of the culture was transferred to 20 liters of the same medium in a 30-liter jar fermenter, which was cultured at 28°C for 1 day under aeration of 20 liters per minute and agitation of 150 rpm. An approximately 6-liter portion of the second culture was transferred to a 250-liter jar fermenter containing 160 liters of a production medium consisting of soluble starch 2.0%, corn steep liquor 2.0%, peptone 1.0%, $CaCO_3$ 0.4% (pH 7.0). Fermentation was carried out at 28°C for 3 days under aeration of 160 liters per minute and agitation of 120~150 rpm.

Isolation and Purification

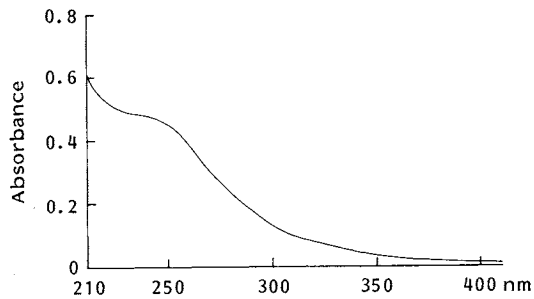
Most of the antibiotic activity was found in the mycelium. The fermented broth (*ca.* 150 liters) was centrifuged to obtain the mycelial mass. The mass was extracted with 50% aqueous acetone (25 liters) twice. The extract was concentrated *in vacuo* to *ca.* 25 liters. Water (10 liters) was added to the concentrate and the solution was extracted twice with ethyl acetate (12 liters) at pH 3.0. When the extract was washed with water and concentrated to *ca.* 200 ml, the antibiotic was precipitated, giving a crude powder (7.72 g).

The crude powder (1.5 g) obtained as above was dissolved in 40 ml of chloroform - methanol (20 : 1). The solution was washed with dil HCl and water, then dehydrated with magnesium sulfate and concentrated to a residue. It was applied to silica gel plates (Merck, Silica gel 60 F_{254}) and developed with chloroform - methanol (9 : 1). The zone (Rf *ca.* 0.35) of the antibiotic detectable by a UV lamp was extracted with chloroform - methanol (8 : 2). The extract was concentrated and diluted with chloroform. The solution was washed with dil HCl and water, dehydrated and concentrated to give the antibiotic as a colorless powder (250 mg).

Physico-chemical Properties

Thioxamycin is acidic in nature. The free acid is obtained as a colorless powder, mp *ca.* 260°C (dec); $[\alpha]_D^{25} -67.7°$ (*c* 0.5, $CHCl_3$ - MeOH). The antibiotic is soluble in tetrahydrofuran, chloroform and dimethyl sulfoxide, slightly soluble in methanol and ethyl acetate, but substantially insoluble in petroleum ether and water. It is sensitive to oxygen and has a tendency to degenerate to an inactive material which is insoluble in common organic solvents. It shows positive reaction with Dragendorff reagent and decolorizes potassium permanganate, but negative reactions with ninhydrin and Sakaguchi reagents. The UV spectrum (Fig. 2) shows a shoulder at 252 nm ($E_{1\,cm}^{1\%}$ 440). Dominant absorptions at 1662 and 1520 cm^{-1} in the IR spectrum (Fig. 3) indicated the antibiotic to be a peptide.

Fig. 2. UV absorption spectrum of thioxamycin in MeOH.



A molecular formula, $C_{52}H_{48}N_{16}O_{15}S_4$, was indicated by secondary ion (SI)-MS (*m/z* 1,265 $(M+H)^+$) and elemental analysis.

Fig. 3. IR absorption spectrum of thioxamycin (KBr).

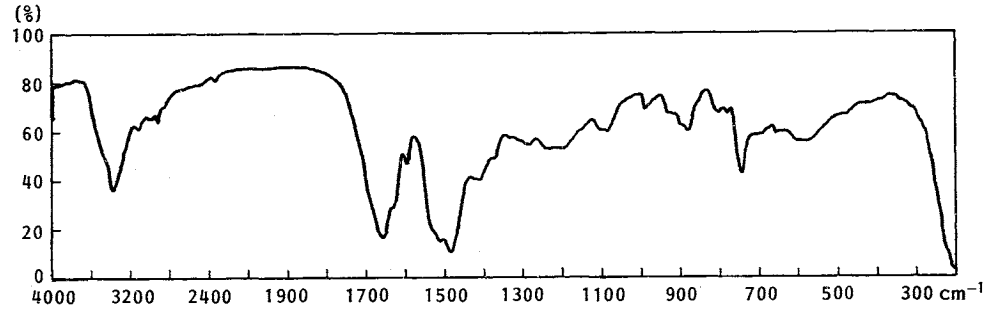


Table 3. Antibacterial spectrum of thioxamycin against anaerobic bacteria.

Test organism	MIC (μg/ml)
Peptococcus asaccharolyticus ATCC 14963	6.25
P. prevotii ATCC 9321	12.5
Peptostreptococcus micros VPI 5464-1	12.5
Streptococcus constellatus ATCC 27823	6.25
Eubacterium limosum ATCC 8486	1.56
E. aerofaciens ATCC 25986	3.13
Propionibacterium acnes ATCC 11827	100
Bifidobacterium adolescentis JCM 1250	1.56
B. bifidum JCM 1122	0.78
B. longum ATCC 15707	0.78
Clostridium perfringens ATCC 13124	0.39
C. difficile ATCC 17857	3.13
Veillonella parvula ATCC 10790	>100
Bacteroides fragilis GM 7000	25
B. fragilis ATCC 25285	12.5
B. thetaiotaomicron WAL 3304	25
B. vulgatus ATCC 29327	6.25
B. melaninogenicus GAI 0413	6.25
Fusobacterium varium ATCC 8501	>100
F. necrophorum ATCC 25286	25
F. nucleatum ATCC 25586	12.5
F. mortiferum ATCC 9817	>100

Inoculum size: One loopful of 10^6 cfu/ml.
Medium: GAM Agar (Nissui).

Anal Calcd for $C_{52}H_{48}N_{16}O_{15}S_4 \cdot 4H_2O$:
C 46.70, H 4.22, N 16.76, S 9.59.
Found: C 46.84, H 3.79, N 16.48, S 9.55.

When thioxamycin was hydrolyzed with 6 N HCl at 110°C for 20 hours, it gave one mol of threonine and three unusual amino acids (U-1, U-2 and U-3) detected by an automatic amino acid analyzer (Fig. 4) and an acid insoluble substance. The structures of these substances were elucidated to be *S*-methyl-L-cysteine, 2-aminomethylthiazole-4-carboxylic acid, 2-(1-aminoethyl)thiazole-4-carboxylic acid, and berninamycinic acid, respectively, as will be shown in the following paper[3].

Fig. 4. Amino acid analysis of the hydrolysate of thioxamycin.

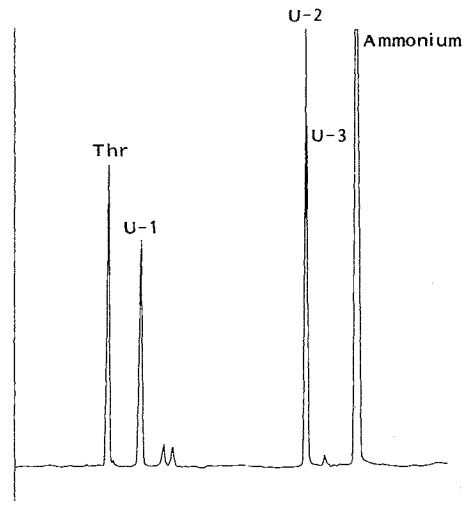


Table 4. Antibacterial spectrum of thioxamycin against aerobic bacteria.

Test organism	MIC (μg/ml)
Staphylococcus aureus FDA JC-1	3.13
S. aureus Smith	3.13
Streptococcus pyogenes C-203	0.39
S. pneumoniae Type 1	0.78
Escherichia coli NIHJ JC-2	>100
E. coli EC-14	>100
Klebsiella pneumoniae SR1	>100
Proteus vulgaris CN-329	>100
Enterobacter cloacae ATCC 13047	>100
Serratia marcescens ATCC 13880	>100
Pseudomonas aeruginosa ATCC 25619	>100

Biological Properties

Thioxamycin is active against a wide variety of species of anaerobic bacteria as shown in Table 3. It also shows activity against aerobic Gram-positive bacteria as illustrated in Table 4.

In an experiment to examine inhibition of incorporation of radioisotopic diaminopimelic acid and leucine into acid-insoluble fraction of a *Bacillus* strain[5], protein synthesis-inhibition was suggested (data not shown) as the mode of action.

No toxic sign was observed following ip administration at a dose of 50 mg/kg to mice.

Experimental

The UV absorption spectra were measured with a Hitachi 323 spectrometer, IR absorption spectra with a Jasco DS-403G spectrometer, $[\alpha]_D$ with a Perkin-Elmer 241 polarimeter and SI-MS with a Hitachi M-90 mass spectrometer. Amino acid analysis was carried out with a Hitachi amino acid autoanalyzer 835.

Acknowledgments

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