TETRANACTIN, A NEW MITICIDAL ANTIBIOTIC

I. ISOLATION, CHARACTERIZATION AND PROPERTIES OF TETRANACTIN

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In our screening for pesticidal antibiotics using Azuki-bean weevil as a test insect, a new antibiotic, tetranactin, was isolated as crystalline rhombic prisms from the filter cake of the fermented broth of *Streptomyces aureus* strain S-3466. Tetranactin shows significant insecticidal activity against Azuki-bean weevil by the topical application method but lucks the activity by the film contact method. The adults of carmine mite are highly sensitive to the antibiotic, since LC_{50} for the mite is 9 ppm by the spray method. The antibiotic inhibits the growth of gram-positive bacteria and some phytopathogenic fungi *in vitro* at low concentrations. Acute toxicity of tetranactin is low; mice tolerated an intraperitoneal administration of 300 mg/kg and an oral administration of 15,000 mg/kg.

A number of reports have appeared which dealed with pesticidal activity of antibiotics. It was found that aureothin[1], antimycin A[2], piericidine[3] and cycloheximide[4] exert pesticidal activity, although the usefulness is strictly limited because of the high oral toxicity to a warm-blooded animal.

In our screening for pesticidal antibiotics using Azuki-bean weevil as a test insect, a crystalline active principle effective against the carmine mite, *Tetranychus telarius*, was isolated from the filter cake of the fermented broth of a soil *Streptomyces* strain S-3466. The active principle was named tetranactin, as it has the same chemical structure to that of a hypothetic compound, tetranactin, described by GERLACH *et al*[5]. This paper deals with isolation, characterization and some properties of tetranactin and identification of the producing microorganism.

Identification of Producing Microorganism

The producing organism, *Streptomyces* strain S-3466, was isolated from the soil collected at Tsurugashima, Saitama Prefecture, Japan. Taxonomy was carried out according to WAKSMAN[6]. Morphological characteristic of the strain was as follows: sporophres long, straight or wavy, but not forming spirals; spherical to oval, 0.7~0.8 $\mu \times 1.0$ μ. Morphological characteristics on various media are listed in Table 1. The strain S-3466 is closely related to *Streptomyces aureus* WAKSMAN *et* HENRICI, although the characteristics are slightly different from those described by WAKSMAN[6].

Table 1. Morphological characteristics of *Streptomyces aureus* S-3466 on various media

Medium	Growth	Aerial mycelium	Soluble pigment	
Sucrose nitrate agar	Thin	Thin, powdery, cream colored	None	
Malate glycerol agar	Good, cream colored	Powdery, yellowish white	Pale brown	
Glucose-asparagine agar	Good, pale yellow	Powdery, yellowish white	Pale yellow	
Glycerol-asparagine agar	Good	Powdery, pale yellow	Pale yellowish brown	
Nutrient agar	Good	Buff colored	Pale yellowish brown	
BENNETT's agar	Good wrinkled, raised center	Yellowish gray	Dark yellowish brown	
Starch agar	Moderate, colorless	Yellowish white	Pale yellowish orange	Hydrolysis: good
Potate plug	Abundant, wrinkled, yellowish brown	Light brownish gray		
Nitrate	Flocculent			Reduction to nitrite
Gelatin agar	Surface growth moderate	White	Brown	Liquefaction: slow Crateriform
Litmus milk	Good, ring			Coagulation and peptonization: weak
Starch peptone beef agar	Good, wrinkled	Pale yellowish brown	Dark yellowish brown	
Dextrin casein digest agar	Wrinkled, yellowish brown	Brownish white	Dark yellowish brown	
Cellulose-asparagine	None			Hydrolysis: none
Invertase				Negative

The strain S-3466 was compared with *S. aureus* IAM 0092, an original strain isolated by WAKSMAN and deposited in American Type Culture Collection (ATCC 3309).

Production and Isolation of Tetranactin

In our screening for pesticidal antibiotics, soil streptomyces strains isolated were aerobically grown for 4 days at 28°C in a medium composed of (w/v, %); glucose 2, soybean meal 2, glycerol 2, peptone 0.2, yeast extract 0.2, beaf extract 0.2, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.05 and $CaCO_3$ 1 (pH, not adjusted). Acetone extracts of the filter cake of the fermented broth were directly applied to the test insect, Azuki-bean weevil (*Callosobruchus chinensis*). An extract of streptomyces strain S-3466 exerted significant and reproducible insecticidal activity in the screening. The extract showed several spots when subjected to preparative thin-layer chromatography, but only one spot (Rf 0.6) possessed insecticidal activity.

Fractionation and isolation of the active principle is shown in Fig. 1. *S. aureus* strain S-3466 was aerobically grown for 3 days at 28°C in a jar fermentor of 100-liter volume containing 60-liter medium. The medium composition was the same as in the screening. The jar was aerated at a rate of 30 liters/minute and agitated at 250 r.p.m.

The fermented broth was filtered with Celite. The filter cake obtained was extracted with acetone overnight at room temperature. The acetone extract (15 liters) was concentrated *in vacuo* to remove acetone and the resulting suspension was re-extracted with ethylacetate (2×3 liters). The ethylacetate layer was dehydrated with anhydrous sodium sulfate and then concentrated *in vacuo* to a tarry brown residue (120 g) which represented more than 90% of the insecticidal activity present in the original acetone extract. The active principle was isolated by chromatography of the tarry oil on a 3 cm×75 cm silica gel column. The column was first eluted with *n*-hexane (1 liter) yielding an oily residue which was largely triglyceride and was inactive against the test insect. Then the column was eluted with a mixture of *n*-hexane and acetone (1 : 1, 1 liter). Fatty acids recovered from the eluate were also inactive against the test insect. Finally the column was eluted with ethylacetate (1 liter) to yield a colorless oil with insecticidal activity (18 g). The oil crystallized as large rhombic prisms from *n*-hexane at −10°C. However, thin-layer chromatography on silica gel (Table 2) indicated the presence of three components even after repeated recrystallization. The main component with the lowest Rf was named tetranactin. Tetranactin was separated from the other two components by silica gel column chromatography as shown in Fig. 2.

Fig. 1. Extraction and isolation of tetranactin

Azuki bean weevil, *Callosobruchus chinensis*, was used as a test insect during the course of isolation. Dipping method was used for activity check.

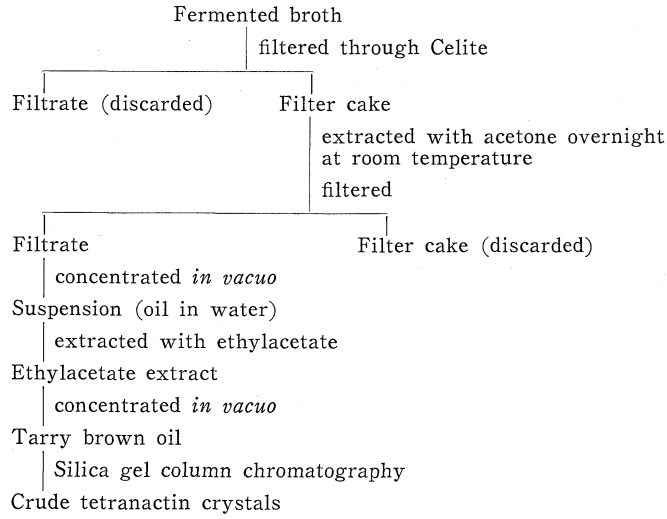


Fig. 2. Separation of tetranactin from two other macrotetrolides

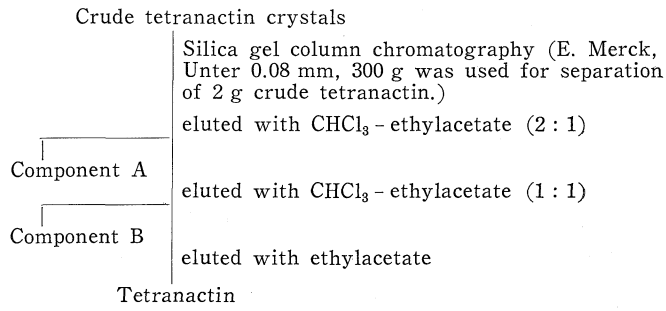


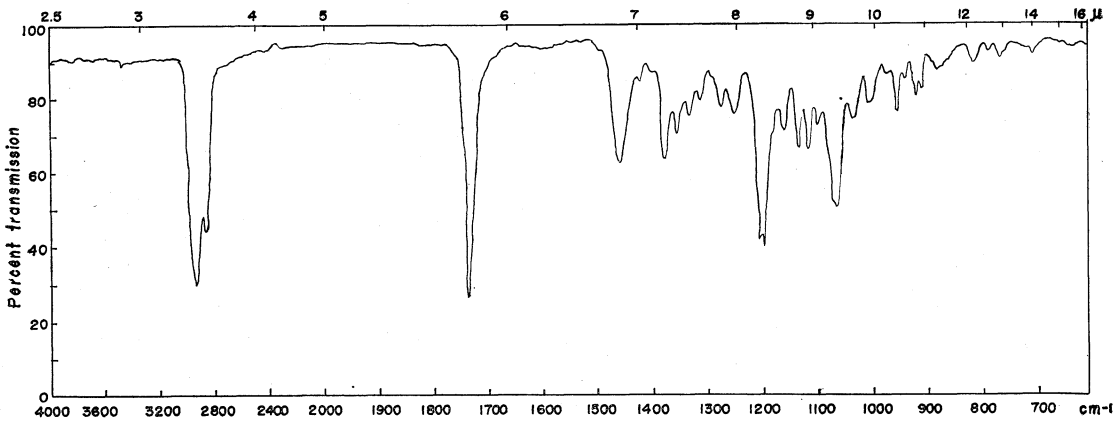
Table 2. Rf values of each component on thin-layer chromatograms.

Solvent systems	Rf of each component		
	Component A	Component B	Tetranactin
Benzene - acetone (4 : 1)	0.37	0.25	0.12
$CHCl_3$ - ethylacetate (1 : 2)	0.60	0.48	0.31
n-Hexane - diethylether (1 : 2)	0.42	0.32	0.21

Physicochemical Properties of Tetranactin

Tetranactin readily crystallized as rhombic prisms from acetone, m.p. 105~106°C, $[\alpha]_D^{23.5}$ 0° (*c* 1, chloroform). The following reactions were all negative; FEHLING, TOLLENS, carbazole, ELSON-MORGAN and ninhydrin. The antibiotic is readily soluble in most organic solvents such as *n*-hexane, cyclohexane, benzene, chloroform, diethylether, acetone, ethylacetate and alcohols, but insoluble in water. Elementary analysis showed C 66.75 %, H 9.13 %; halogens, nitrogen and sulfur were not detected. The antibiotic showed no ultraviolet absorption maxima in the region of 200~400 mμ. Infrared absorption (IR) spectrum is shown in Fig. 3. The IR spectrum indicated the presence of ester and ether as functional groups which are characteristic for the class macrotetrolide antibiotic. The IR spectrum closely resembles that of nonactin[7], the first macrotetrolide isolated. However, tetranactin is distinguishable from nonactin and other macrotetrolides[7,8] by its physicochemical properties.

Fig. 3. Infrared absorption spectrum of tetranactin (nujol).



Biological Properties of Tetranactin

Table 3 shows the insecticidal activity of tetranactin against the female adults of Azuki-bean weevil, an insect used in the screening. Mortality was 100 % at a dose of 1.5 μg/insect when topically applied. However, tetranactin was inactive in the filter paper method, indicating neither vapor nor penetration through legs to active sites were involved in the insecticidal activity. For insecticidal activity tetranactin must be directly applied to the insect.

Table 4 shows the miticidal activity of tetranactin and the other two components against the adults of carmine mite. The LC_{50} values calculated are 9.6 ppm for component A,

Table 3. Insecticidal activity of tetranactin against the female adults of Azuki-bean weevil, *Callosobruchus chinensis*

Topical application method	Dose (μg/insect)			
	0.25	0.50	1.00	1.50
Mortality (%)	0.0	10.3	84.8	100.0

Application method	Dose	Mortality (%)
Filter paper method	2 mg/filter paper	0
Film contact method	2 mg/petri dish	45

Fifty to a hundred of the female adults of *Callosobruchus chinensis* (English, Azuki-bean weevil. Japanese, Azuki zomushi) were used in each dose. Diameters of filter paper and petri dish were 9 cm. Mortality was observed 48 hours after applications.

10.3 ppm for component B and 9.3 ppm for tetranactin. These values are comparable to those of synthetic organic miticides.

Table 4. Miticidal activity of tetranactin and structurally related macrotetrolides

	Mortality (%) at each concentration (μg/ml)					LC_{50} (μg/ml)
	40	20	10	5	2.5	
Tetranactin	86.5	75.9	52.5	33.6	32.0	9.2
Component A	81.5	72.7	46.5	39.4	34.8	9.6
Component B	76.0	65.1	50.9	37.2	43.5	10.8

Fifty to a hundred adults of carmine mite, *Tetranychus telarius*, were used at each concentration. The aqueous suspensions of the three were sprayed onto the leaves of the host plant, kidney bean, with the mites and mortality was observed 48 hours after spray.

As can be seen in Tables 5 and 6, tetranactin inhibits the growth of gram-positive bacteria and phytopathogenic fungi by the agar dilution method. The antibiotic, however, formed very small growth inhibitory zones (<12 mm) in the agar diffusion method at a dose of 200 μg/disc. It is likely that the small growth inhibitory zones formed by tetranactin are due to the insolubility in water. Gram-negative bacteria except *Shigella sonnei* were insensitive to

Table 5. Antimicrobial spectrum of tetranactin

Microorganisms tested	Minimum inhibitory concentration (μg/ml)
Arthrobacter simplex	0.9
Brevibacterium ammoniagenes	0.1
Microbacterium flavum	0.9
Micrococcus flavus	0.9
Serratia marcescens	>24.3
Staphylococcus aureus	< 0.1
Sarcina lutea	0.3
Bacillus megaterium	0.9
Bacillus cereus	0.9
Bacillus subtilis	0.9
Bacillus roseus	0.9
Bacillus circulans	0.1
Bacillus firmus	0.9
Shigella sonnei	< 0.1
Aerobacter aerogenes	>24.3
Agrobacter radiobacter	>24.3
Alcaligenes faecalis	>24.3
Escherichia coli	>24.3
Salmonella typhosa	>24.3
Klebsiella pneumoniae	>24.3
Proteus vulgaris	>24.3
Pseudomonas aeruginosa	>24.3
Pseudomonas ovalis	>24.3
Xanthomonas oryzae	>24.3
Xanthomonas citri	>24.3
Xanthomonas phaecalis	>24.3
Erwinia aroidea	>24.3
Corynebacterium michiganensis	>24.3
Mycobacterium tuberculosis	8.1
Mycobacterium 607	>24.3

The growth was observed 72 hours after inoculation on nutrient broth agar plate except *Mycobacterium*. *Mycobacterium* was grown on KIRCHNER medium and the growth was observed 14 days after inoculation.

Table 6. Antimicrobial spectrum of tetranactin against fungi.

Fungi tested	Minimum inhibitory concentrations (μg/ml)
Candida albicans	>24.3
Candida krusei	8.1
Cryptococcus neoformans	>24.3
Hansenula anomala	24.3
Saccharomyces cerevisiae	>24.3
Cercospora oryzae	>24.3
Gibberella saubinetii	8.1
Gibberella fujikuroi	>24.3
Botrytis cinerea	24.3
Botrytis tulipae	>24.3
Sclerotinia arachidis	>24.3
Fusarium lini	>24.3
Cochliobolus miyabeanus	0.9
Macrosporium bataticola	8.1
Alternaria kikuchiana	>24.3
Corynespora vignicola	>24.3
Glomerella cingulata	>24.3
Glomerella lagenarium	>24.3
Cladosporium fluvum	>24.3
Colletotrichum atromentarium	>24.3
Rosedlinis necatrix	>24.3
Piricularia oryzae	2.7
Pellicularia sasakii	8.1
Gloeosporium kaki	>24.3
Helminthosporium sesanum	>24.3
Rhizoctonia solani	0.9
Phoma citricarpa	>24.3
Pestalotia diospyri	>24.3
Mycospherella arachidicola	>24.3
Aspergillus oryzae	>24.3
Aspergillus fumigatus	>24.3
Penicillium citrinum	>24.3
Aspergillus niger	>24.3

tetranactin. It is noteworthy that *S. sonnei* resistant to streptomycin, chloramphenicol, tetracyclines and sulfonamides is inhibited at less than 0.03 μg/ml.

Acute toxicity of tetranactin is low; the LD_{50} is >300 mg/kg (mice, i.p.) and >15,000 mg/kg (mice, p.o.). Thus, tetranactin is a potential miticidal agent in view of the low toxicity to a warm-blooded animal and remarkable activity.

Elucidation of the structure and evaluation of the miticidal activity will be presented in subsequent papers.

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