Gualamycin, a Novel Acaricide Produced by *Streptomyces* sp. NK11687

I. Taxonomy, Production, Isolation, and Preliminary Characterization†

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(Received for publication February 27, 1995)

A novel acaricide, gualamycin, was isolated from the culture broth of *Streptomyces* sp. NK11687. It was purified from the filtrate by column chromatographies. Gualamycin showed 100% acaricidal activity at 250 μg/ml against sensitive and resistant mites to Dicofol.

It is a serious problem for agriculture that mites have become resistant to many acaricides. In our screening of acaricides in microbial secondary metabolites, we found a novel acaricide from the culture broth of *Streptomyces* sp. NK11687 (Fig. 1). It showed good cidal activity against sensitive mites as well as resistant mites to Dicofol. In this report, we describe the taxonomy, production, isolation and physico-chemical and biological properties of gualamycin. The structural elucidation by spectral analyses is reported in the proceeding paper[1].

Materials and Methods

Taxonomy

The producing organism, NK11687, was isolated from a soil sample collected at Ageo, Saitama, Japan. The media and procedures used for the cultural and physiological characterization of strain NK11687 were according to the methods of SHIRLING and GOTTLIEB[2]. Each culture was incubated at 27°C for 2 weeks before observation. The color names used in these studies were based on the Color Standard of Nihon Shikisai Co. Ltd.. Chemical composition of the cells was determined using the methods of BECKER *et al.*[3] and YAMAGUCHI[4]. Detailed observations of mycelial and spore morphologies were performed with a light microscope and a scanning electron microscope (JEOL 25S III).

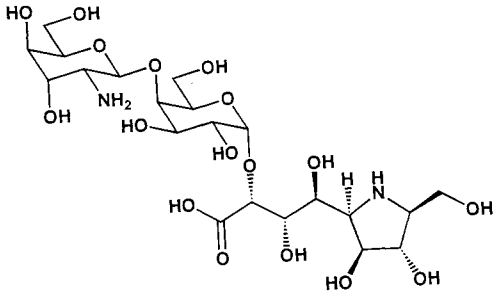
Production

A loopful spores of the strain NK11687 was inoculated into 100 ml of a production medium consisted of galactose 2%, dextrin 2%, Bacto-Soytone (DIFCO) 1%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% (pH 7.4 before sterilization) in 500-ml Erlenmeyer flask, and cultured at 27°C for 2 days on a rotary shaker (220 rpm). One milliliter of the seed culture was transferred to 100 ml of the same medium in a 500-ml Erlenmeyer flask and cultured at 27°C for 5 days.

Isolation and Purification

The fermentation broth (20 liters) was separated into filtrate (19 liters) and mycelia by filtration. The filtrate was applied on a column of active charcoal (2.4 liters), and gualamycin was eluted with a linear gradient of aqueous methanol from 0 to 100% after washing the column with water. The active fractions were collected and evaporated to aqueous solution. This solution was absorbed on a column of Dowex 50W (H^+, 100 ml). The column was washed with water and then eluted with 2.8% aqueous ammonia. The active fractions were collected, concentrated and lyophilized. The resulting powder was dissolved in a small volume of water and applied on a column of CM-Sephadex C-25 (Na^+,

Fig. 1. Structure of gualamycin.



† Dedicated to Prof. S. ŌMURA on his 60th birthday.

900 ml) and eluted with a stepwise gradient of aqueous sodium chloride. The active fractions were collected and desalted by Micro Acilyzer S1 (Asahi Chemical Industry Co., Ltd.) to give pure gualamycin (1.72 g).

Acaricide Assay

The test method for acaricidal (adult) activity:

First two leaves of dwarf kidney bean grown in a porous pot were cut into squares (3 cm × 3 cm), and dipped into each test solution (50% aqueous ethanol). Approximate 50 heads of adult female mites were infested on the leaves after drying, and kept at 25°C during the test period. The numbers of alive and dead mites were counted after 3 days under a binocular microscope for determination of the mortality.

The test method for acaricidal (larvae) activity:

Approximately 10 heads of adult female mites were released on a cutting leaf (3 cm × 3 cm) and allowed to oviposit on it for 24 hours. After removing the adult mites, the leaf having their eggs was dipped into the test solution and kept at 25°C for 10 days. The mortality of larvae was determined by the same method as for the adult mites.

Results and Discussion

Taxonomy of Strain NK11687

Morphological observations were made with light and scanning electron microscopes (Fig. 2) on the culture grown at 27°C for 2 weeks on inorganic salts-starch agar. This strain showed spiral or hooked hyphae from branched aerial hyphae and no whirl. No sporangia, zoospores, vegetative mycelium spore or synnemata was observed. A matured spore chain comprised 20 or more spores (0.7~0.9 × 0.9~1.4 μm) with a smooth surface. The cultural characteristics of strain NK11687 are summarized in Table 1. The aerial mass color was brownish white to pale pink. A slight brown soluble pigment was observed without melanoid pigment. The physiological characteristics of strain NK11687 are summarized in Table 2. Hydrolyzed whole-cell of strain NK11687 contained LL-diaminopimelic acid. Accordingly, the cell wall of this strain was determined to be Type I. Based on the taxonomy described above, and judgement to be a strain of the red series of the PRIDHAM and TRESNER grouping[5], the strain NK11687 is considered to belong to the genus *Streptomyces*. The strain NK11687 was compared with *Streptomyces* species described in the literatures[5~12]. The strain NK11687 has been deposited in the National Institute of Bioscience and Human-Technology (formerly The Fermentation Research Institute), Agency of Industrial Science and Technology, Japan under the accession No. FERM P-13207.

Production and Isolation

The fermentation broth was separated into filtrate (19

Fig. 2. Scanning electron micrograph of a spore chain of strain NK11687 on inorganic salts-starch agar at 27°C for 2 weeks culture.

Bar represents 1 μm.

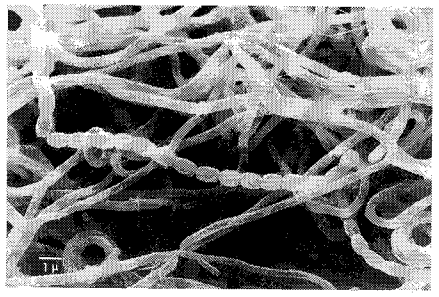


Table 1. Cultural characteristics of strain NK11687.

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Sucrose-nitrate agar	Good	Yellowish white~pale olive	Pale yellowish brown	Brown
Glucose-asparagine agar	Good	Light brownish gray~brownish gray	Pale yellowish brown~yellowish brown	Faint, yellow
Yeast extract-malt extract agar (ISP medium 2)	Good	Brownish white~light brownish gray	Pale yellowish brown	Faint, brown
Oatmeal agar (ISP medium 3)	Good	Light brownish gray~brownish gray	Yellow~pink	Faint, yellow
Inorganic salts-strach agar (ISP medium 4)	Good	Brownish white~brownish gray	Pale yellowish brown~yellowish brown	Faint, yellow
Glycerol-asparagine agar (ISP medium 5)	Good	Light brownish gray	Pale yellowish brown~yellowish brown	Faint, yellow
Tyrosine agar (ISP medium 7)	Good	Light brownish gray	Pale yellowish brown~yellowish brown	Faint, brown
Nutrient agar	Good	White	Colorless~pale yellow	None

The observed was carried out after incubation at 27°C for 2 weeks.

Table 2. Physiological characteristics of strain NK11687.

Temperature range for growth (°C)	10~37	Utilization of[a]	
Optimum temperature (°C)	27~37	D-Glucose	+
Reduction of nitrate	−	L-Arabinose	+
Hydrolysis of strach	+	D-Xylose	+
Coagulation of milk	−	D-Fructose	+
Peptonization of milk	+	Sucrose	+
Melanoid pigment		L-Rhamnose	+
Tyrosine agar	−	Inositol	+
Peptone - yeast extract - iron - agar	−	D-Mannitol	+
Tryptone - yeast extract broth	−	Raffinose	+
Liquefaction of gelatin	+	D-Galactose	+

+; Positive, −; negative.
[a] Basal medium: PRIDHAM-GOTTLIEB's carbon utilization medium.

Fig. 3. Isolation of gualamycin.

Whole broth (20 liters)
| filtered
Filtrate (19 liters)
| active charcoal column chromatography
| eluted with linear gradient of 0 ~100% aqueous MeOH
Eluate (20~40% aqueous MeOH fractions)
| evaporated to aqueous solution
| Dowex 50W X 2 (H^+ type) column chromatography
| eluted with 2.8% aqueous NH_4OH
Active fractions
| concd. *in vacuo*
| CM-Sephadex C-25 (Na^+ type) column chromatography
| eluted with a stepwise gradient of NaCl in water
Active fractions
| concd. *in vacuo*
| desalted by MICRO ACILYZER S1
| lyophilized
White powder of gualamycin (1.72g)

Table 3. Physico-chemical properties of gualamycin.

Appearance	White powder
MP	174°C
$[\alpha]_D^{20}$	+49.0° (*c* 0.1, H_2O)
FAB-MS (*m/z*)	591 $(M+H)^+$
HRFAB-MS (*m/z*)	591.2288 $(M+H)^+$ (3.9 m.m.u.) (Calcd for $C_{21}H_{39}N_2O_{17}$ *m/z* 591.2249)
Molecular formula	$C_{21}H_{38}N_2O_{17}$
UV	End absorption (H_2O)
IR λ_{max} (KBr) cm^{-1}	3412, 1607
Rf* value	0.3
Solubility	Soluble in H_2O, DMSO Insoluble in MeOH, EtOH, Me_2CO, EtOAc

Rf*: Silica gel TLC (Kieselgel 60F 0.25 mm, Merck) was used with developing soluvent BuOH - EtOH - $CHCl_3$ - 28% NH_4OH (4:5:2:8).

Table 4. Acaricidal activities of gualamycin.

Compound	Conc. (μg/ml)	Mortality (%)			
		Sensitive		Resistant**	
		Adult	Larvae	Adult	Larvae
Gualamycin	1,000	100	100	100	100
	500	100	100	100	100
	250	100	100	100	100
	125	80	30	80	50
	62	50	0	80	10
	31	0	0	0	0
Dicofol*	1,000	100	100	80	50
	500	100	100	50	30
	250	100	100	10	10
	125	80	100	0	0
	62	50	50	0	0
	31	0	0	0	0

* Dicofol: 2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol.
** Mites that are resistant to Dicofol.

liters) and mycelia by filtration. Gualamycin (1.72 g) was isolated from the filtrate according to the isolation procedure summarized in Fig. 3.

Physico-chemical Properties

The physico-chemical properties of gualamycin are summarized in Table 3. Gualamycin is soluble in water and dimethyl sulfoxide, but insoluble in methanol, ethanol, acetone and ethyl acetate. The spot on silica gel TLC plate was visualized by ninhydrin. The structure of gualamycin (Fig. 1) was elucidated on the basis of physico-chemical properties (Table 3), ^1H and ^{13}C NMR spectrometric, X-ray crystallographic analyses and synthetic studies. The details of these studies will be reported in the proceeding paper[1].

Biological Activities

Gualamycin showed potent acaricidal activities without any phytotoxicities of kidney bean. The activities of gualamycin are shown in Table 4. Gualamycin showed 100% acaricidal activities against both adults and larvae at the dose of 250 μg/ml. Therefore gualamycin showed the superior acaricidal activity to Dicofol (the commercial acaricide) against resistant mites to the Dicofol,

and both gualamycin and Dicofol showed the same level of activities against the sensitive mites.

References

1) TSUCHIYA, K.; S. KOBAYASHI, T. KUROKAWA, T. NAKAGAWA, N. SHIMADA, H. NAKAMURA, Y. IITAKA, M. KITAGAWA & K. TATSUTA: Gualamycin, a novel acaricide produced by *Streptomyces* sp. NK11687. II. Structural elucidation. J. Antibiotics 48: 630~634, 1995
2) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
3) BECKER, B.; M. P. LECHVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
4) YAMAGUCHI, T.: Comparrison of the cell-wall composition of morphologically distinct actinomycetes. J. Bacteriol. 89: 444~453, 1965
5) PRIDHAM, T. G. & H. D. TRESNER: Genus I. *Streptomyces* Waksman and Henrici 1943. *In* BERGEY's Manual of Determinative Bacteriology, 8th Ed. *Eds.*, R. E. BUCHANAN & N. E. GIBBONS, pp. 748~829, Wilkins Co., 1974
6) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriol. 56: 107~114, 1948
7) NONOMURA, H.: Key for classification of 458 species of *Streptomyces* included in ISP. J. Ferment. Technol. 52: 78~92, 1974
8) LECHEVALIER, M. P. & H. A. LECHEVARIER: Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 20: 435~443, 1970
9) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Int. J. Syst. Bacteriol. 18: 69~189, 1968
10) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. III. Addditional Species descriptions from first and second studies. Int. J. Syst. Bacteriol. 18: 279~392, 1968
11) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from second, third and fourth studies. Int. J. Syst. Bacteriol. 19: 375~390, 1969
12) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. V. Additional descriptions from first study. Int. J. Syst. Bacteriol. 22: 265~394, 1972