MELANOSTATIN, A NEW MELANIN SYNTHESIS INHIBITOR

PRODUCTION, ISOLATION, CHEMICAL PROPERTIES, STRUCTURE AND BIOLOGICAL ACTIVITY

YASUMASA ISHIHARA, MASAHISA OKA, MITSUAKI TSUNAKAWA, KOJI TOMITA, MASAMI HATORI, HARUAKI YAMAMOTO, HIDEO KAMEI, TAKEO MIYAKI, MASATAKA KONISHI and TOSHIKAZU OKI

Bristol-Myers Squibb Research Institute,
2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan

(Received for publication July 14, 1990)

Melanostatin, a new antibiotic with melanin synthesis inhibitory activity, was isolated from the fermentation broth of *Streptomyces clavifer* No. N924-2. Its structure was determined by spectral analysis and degradation experiments. Melanostatin strongly inhibited melanin formation in *Streptomyces bikiniensis* NRRL B-1049 and B16 melanoma cells.

In a previous paper[1], we reported a screening method for melanin synthesis inhibitors among microbial metabolites and the isolation of several known active substances obtained from such screening.

In our continuing search for the active metabolites, a microbial organism isolated from a soil sample collected in Greece was found to produce an active compound designated melanostatin. Taxonomical study showed that the producing organism, strain N924-2 was *Streptomyces clavifer*. The active principle in the fermentation broth was isolated by use of ion exchange and gel permeation chromatography. Structural studies revealed that melanostatin is a novel pseudotripeptide with a molecular formula of $C_{19}H_{25}N_5O_5$ and is structurally related to feldamycin[2] and FR-900,490[3]. Melanostatin inhibited melanin formation in *Streptomyces bikiniensis* NRRL B-1049 and B16 melanoma cells but did not show inhibitory activity against mushroom tyrosinase. This paper presents the taxonomy of the producing strain No. N924-2 and the fermentation, isolation, structure and biological activity of melanostatin.

Taxonomy

Strain N924-2 was isolated from a soil sample collected in Greece.

Morphology

Strain N924-2 forms branching hyphae (0.5 μm in width), which develop into substrate and aerial mycelia. Both mycelia are well-branched and non-fragmentary. The aerial hyphae are monopodially branched, and bear long straight spore chains. The spore chains contain 10 to 50 or more spores in a chain. The spores are rectangular in shape, $0.4\sim0.5\times0.6\sim0.8$ μm in size, with a smooth surface (Fig. 1). Sclerotium, sporangium and motile spores are not observed.

Cultural and Physiological Characteristics

The sporulated aerial mycelia are white to yellow. The color of substrate mycelia is colorless or moderate yellow to light olive brown. No distinct pigments are produced. Tyrosinase reaction is negative. Gelatin and starch are hydrolyzed. The NaCl tolerance is seen at 9%. The growth range is from 12°C to

Fig. 1. Scanning electron micrographs showing straight spore chains of strain N924-2.

(A) Medium: Inorganic salts-starch agar (ISP medium No. 4). Cultivation: 28°C for 14 days.
(B) Medium: Yeast extract-malt extract agar (ISP medium No. 2). Cultivation: 28°C for 21 days.

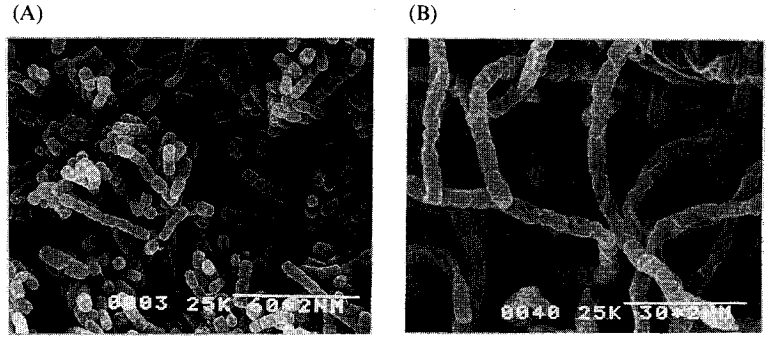


Table 1. Cultural characteristics of strain N924-2.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Sucrose-nitrate agar (CZAPEK-DOX agar)	Poor	Poor; white (263)	Colorless	None
Tryptone-yeast extract broth (ISP No. 1)	Moderate; not turbid	None	Colorless	None
Yeast extract-malt extract agar (ISP No. 2)	Good	Abundant; yellowish white (92)	Light olive brown (94)	Moderate yellowish brown (77)
Oatmeal agar (ISP No. 3)	Moderate	Moderate; yellowish white (92)	Colorless	None
Inorganic salts-starch agar (ISP No. 4)	Good	Abundant; white to yellowish white (92)	Moderate yellow (87)	None
Glycerol-asparagine agar (ISP No. 5)	Good	Abundant; yellowish white (92)	Pale yellow (89)	None
Peptone-yeast extract-iron agar (ISP No. 6)	Moderate	None or scant; white (263)	Colorless	None
Tyrosine agar (ISP No. 7)	Moderate	Moderate; yellowish white (92)	Colorless	None
Glucose-asparagine agar	Moderate	Poor; yellowish white (92)	Colorless	None
BENNETT's agar	Moderate	Moderate; yellowish white (92)	Moderate yellow (87)	None

Observations after incubation at 28°C for 3 weeks.
Color and number in parenthesis follow ISCC-NBS designation.

35°C with no growth observed at 37°C. Among eleven sugars described in BERGEY's Manual[4], D-glucose, D-xylose, L-rhamnose, D-fructose, D-galactose, D-mannitol, salicin and sucrose are utilized for growth, whereas L-arabinose, raffinose and inositol are not utilized. The cultural characteristics are shown in Table 1.

Chemotaxonomy

The whole-cell hydrolysate contains the LL-isomer of diaminopimelic acid and no diagnostic sugar.

Taxonomic Position

The above-mentioned characteristics of strain N924-2 revealed that it belongs to the genus

Streptomyces. According to the descriptions of the genus *Streptomyces* by PRIDHAM and TRESNER[4], the major characteristics of strain N924-2 are summarized as follows: 1) aerial mycelium, white (W) or yellow (Y), 2) spore chain, *Rectus-Flexibilis* (RF), 3) chromogenicity, negative (C−), and 4) spore ornamentation, smooth (SM). This type of *Streptomyces* species is shown in Table 17.41b (W, RF, C−, SM) and Table 17.43b (Y, RF, C−, SM) of BERGEY's Manual, which include 7 species and 46 species, respectively. The morphological, cultural and physiological characteristics of strain N924-2 were compared to the descriptions of 53 species by PRIDHAM and TRESNER[4], SHIRLING and GOTTLIEB[5~8], and WAKSMAN[9]. Strain N924-2 resembles *S. clavifer* (Millard and Burr) Waksman 1953, and *Streptomyces lipmanii* (Waksman and Curtis) Waksman and Henrici 1948 in the formation of long spore chain of *Rectus-Flexibilis*, the absence of distinct pigment formation and the profile of sugar utilization. It is a little different from *S. clavifer* in the absence of brick-red substrate mycelium in CZAPEK's sucrose-nitrate agar, and from *S. lipmanii* in the predominant formation of flexuous spore chain of the latter. However, it forms a club-shaped structure at the tip of the straight spore chain similar to that of *S. clavifer* in CZAPEK's agar. Therefore, strain N924-2 is classified as *S. clavifer* based on the spore chain morphology.

Fermentation

A stock culture of *S. clavifer* strain N924-2 was propagated on an agar slant (ISP No.4) having the composition of soluble starch 1%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, NaCl 0.1%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% and agar 2% at 28°C for 7 days. The mature agar slant was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of seed medium composed of glucose 2%, fish meal 1% and $CaCO_3$ 0.5% (the pH being adjusted to 7.0 before sterilization). The flask was then incubated at 28°C for 3 days on a rotary shaker (200 rpm) and 5 ml portions of the growth were transferred into 500-ml Erlenmeyer flasks containing 100 ml of production medium with the same composition as the seed medium. The fermentation was carried out at 28°C for 8 days using a rotary shaker.

Melanin synthesis inhibitory activity was determined by the paper-disc agar diffusion assay method using *S. bikiniensis* NRRL B-1049 as the test organism[1]. A large scale fermentation was carried out in stainless steel fermenters. The seed culture prepared in twenty 500-ml Erlenmeyer flasks was poured into a 200-liter stainless tank containing 120 liters of the production medium described above. The fermentation was run at 28°C for 8 days with stirring at 250 rpm and aerating at 120 liters/minute. The activity of melanostatin reached a maximum of 50 μg/ml after 6 to 7 days fermentation.

Extraction and Purification

The fermentation broth (200 liters, pH 8.3) was centrifuged with the aid of a Sharples-type centrifuge (Kokusan No. 6). The broth filtrate was adjusted to pH 7.3 with 6 N HCl and stirred for 1 hour with active carbon (Takeda, Shirasagi KLH-250, 2.5 kg) to adsorb the activity. The charcoal was collected by filtration, washed with water (25 liters) and eluted with 80% aqueous acetone (20 liters). The eluate was concentrated to an aqueous solution (1 liter) which was subjected to column chromatography on Amberlite IRC-50 (H^+ form, 1 liter) developed with water (1.5 liters) and then with 2 N NH_4OH (1.1 liters). Concentration of the aqueous ammonia eluate yielded 18 g of brown solid. This was chromatographed on a silica gel column (Merck-Darmstadt Art No. 9385, 820 ml) using dichloromethane-EtOH-14% NH_4OH (9 : 14 : 3) as eluant. The eluate was monitored for melanin synthesis inhibitory activity by the paper-disc assay against *S. bikiniensis* NRRL B-1049[1]. The active fractions were combined and concentrated to dryness *in vacuo* to give 930 mg of yellow solid. This solid was purified on a silica gel column (880 ml) eluted

with BuOH - AcOH - water (4 : 1 : 1). The fractions containing melanostatin were detected by the bioassay and also by TLC (SiO_2, BuOH - AcOH - water (3 : 1 : 2), iodine detection). Melanostatin showed a brown spot at Rf 0.19. The appropriate fractions were concentrated *in vacuo* and the residue was re-chromatographed on a silica gel column (270 ml) using the same solvent system as above. Evaporation of the active fractions yielded melanostatin as a nearly homogeneous solid (300 mg) which was subjected to Sephadex LH-20 chromatography (880 ml) for complete desalting. Elution was carried out with 50% aqueous methanol and the active eluate was concentrated to yield 220 mg of pure melanostatin.

Physico-chemical Properties

Melanostatin was isolated as a white amorphous solid. It was readily soluble in water, methanol and ethanol, slightly soluble in dimethyl sulfoxide and insoluble in acetone, dichloromethane, chloroform and ethyl acetate. It gave yellowish brown and dark gray color reactions after spraying with iodine and sulfuric acid, respectively, on TLC plates. Melanostatin was weakly positive to ninhydrin reaction but was negative to Sakaguchi and anthrone reactions. The antibiotic was assigned a molecular formula of $C_{19}H_{25}N_5O_5$ based on the HRFAB mass spectrum and ^{13}C NMR spectrum (19 carbon signals). Table 2 summarizes the physico-chemical properties of melanostatin. The UV spectrum of melanostatin did not show a maximum above 210 nm and its IR spectrum (Fig. 2) showed broad absorption at around 3300~3100 cm^{-1} (OH or NH) and carbonyl absorptions at 1670~1620 cm^{-1}. The ^1H NMR spectrum in D_2O (400 MHz) is illustrated in Fig. 3.

Table 2. Physico-chemical properties of melanostatin.

Nature	White amorphous powder
MP	Gradually dec over 155°C
$[\alpha]_D^{29}$	+50° (*c* 1.0, H_2O),
	+34° (*c* 1.0, 0.1 N HCl)
Molecular formula	$C_{19}H_{25}N_5O_5$
HRFAB-MS (*m/z*)	Obsd: 404.1941 (MH^+)
	Calcd for $C_{19}H_{26}N_5O_5$: 404.1933
UV (H_2O)	End absorption
IR (KBr) cm^{-1}	3300~3100, 1670 (sh), 1620, 1390
TLC, SiO_2	Rf 0.29[a]
	Rf 0.19[b]

[a] CH_2Cl_2 - EtOH - 14% NH_4OH (4 : 7 : 2).
[b] BuOH - AcOH - H_2O (3 : 1 : 2).

Structural Studies

The physico-chemical and spectral properties stated above resemble those of feldamycin[2] and FR-900,490[3] which were found to have melanin

Fig. 2. IR spectrum of melanostatin (KBr).

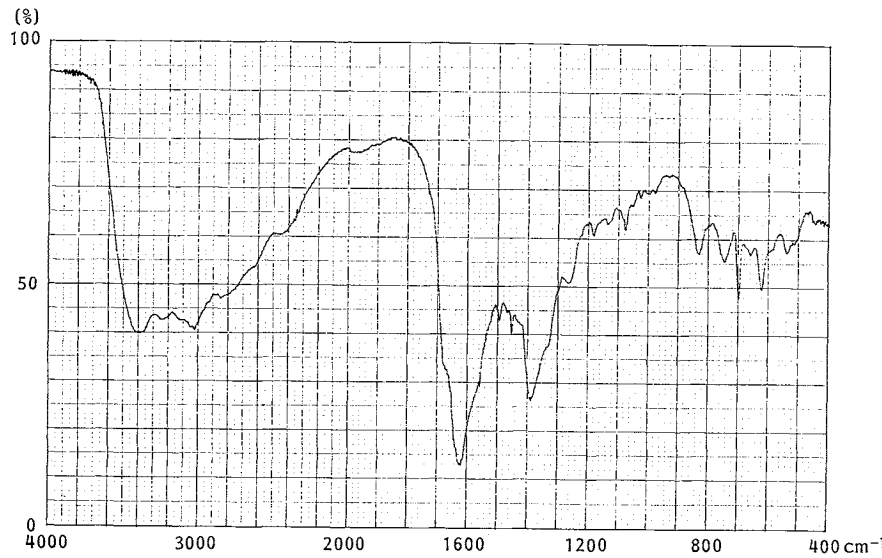


Fig. 3. ^{1}H NMR spectrum of melanostatin (400 MHz in D_2O).

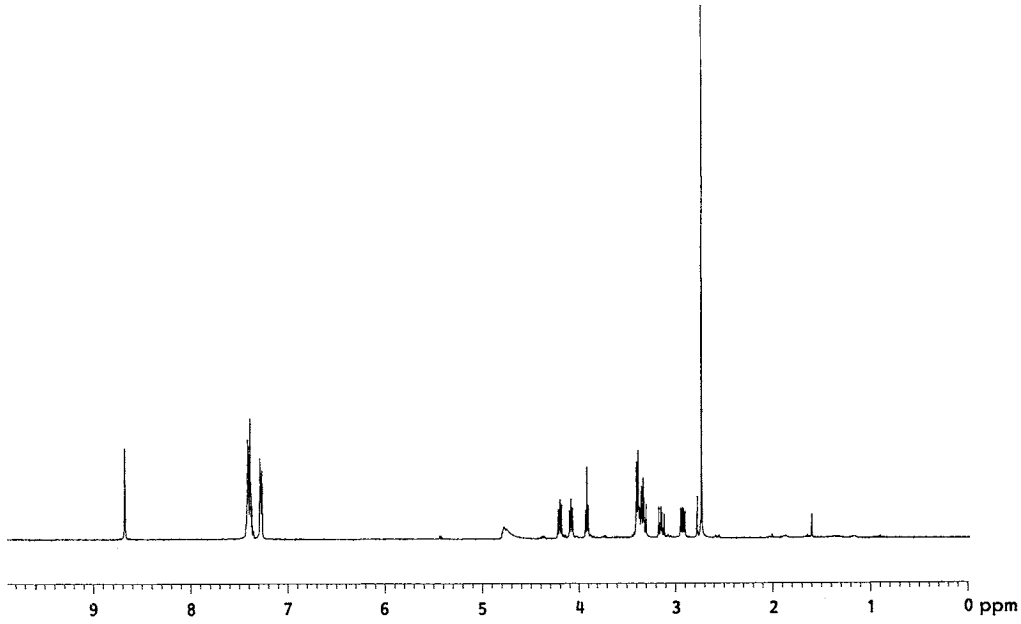


Table 3. ^{1}H NMR spectrum of melanostatin (400 MHz in D_2O).

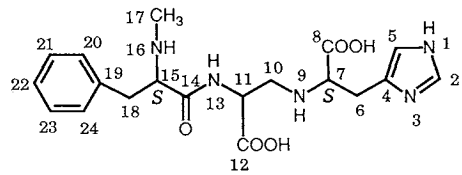
Chemical shift[a]	Interaction and assignment
2.73 (3H, s)	CH_3-N
2.92 (1H, dd, 6.2, 12.2)	
3.14 (1H, dd, 8.6, 13.7)	
3.30 (1H, dd, 7.7, 12.2)	$-CH_2CH<$ 2.92, 3.30 / 4.08
3.35 (1H, dd, 6.4, 13.7)	$-CH_2CH<$ 3.14, 3.35 / 4.20
3.39 (2H, d, 6.6)	
3.91 (1H, t, 6.6)	$-CH_2CH<$ 3.39, 3.91
4.08 (1H, dd, 6.2, 7.7)	
4.20 (1H, dd, 6.4, 8.6)	
7.27 (2H, m)	Phenyl protons (5H)
7.40 (4H, m)	
8.67 (1H, d, 1.5)	Imidazole protons (2H)

[a] δ ppm (intergration, multiplicity, J=Hz).

Table 4. ^{13}C NMR spectrum of melanostatin (100 MHz in D_2O pD=10.5).

Chemical shift[a]	Assignment	Chemical shift[a]	Assignment
31.0 (t)	C-6	129.5 (d)	C-23
33.9 (q)	C-17	130.2 (d)	C-24
39.3 (t)	C-18	130.2 (d)	C-20
49.6 (t)	C-10	133.5 (s)	C-4
56.0 (d)	C-11	136.8 (s)	C-19
64.4 (d)	C-7	137.9 (d)	C-2
65.8 (d)	C-15	176.1 (s)	C-14
119.5 (d)	C-5	177.4 (s)	C-12
127.8 (d)	C-22	181.7 (s)	C-8
129.5 (d)	C-21		

[a] δ ppm (multiplicity).



synthesis inhibitory activity by the *S. bikiniensis* assay system[1]. The molecular formula of melanostatin ($C_{19}H_{25}N_5O_5$) was distinctly different from those of the known antibiotics. The ^{1}H NMR in D_2O exhibited 19 protons which were analyzed with the aid of the ^{1}H-^{1}H COSY spectrum (Table 3) as one $N-CH_3$, one 4-substituted imidazole, one phenyl group and three sets of isolated $CH_2CH<$ groups. On acid hydrolysis in 6 N HCl, melanostatin (30 mg) yielded two ninhydrin-positive substances (AA-1, 11.0 mg and AA-2, 6.4 mg) which were isolated by chromatography on Dowex 50X8. AA-1 was identified as *N*-methylphenylalanine based on its spectral data and comparison with an authentic sample. The L-

configuration was assigned to AA-1 by HPLC comparison with D- and L-*N*-methylphenylalanine using a chiral column (Waters Nova Pak C_{18}, elution: 2 mM *N*,*N*-dipropyl-L-alanine and 1 mM cupric acetate in water, pH 5.0)[10]. AA-2 was identical in all respects with L-histidine[11]. Assignment of the configuration was confirmed by HPLC with a chiral column (Tosoh MFG Co., Ltd., TSK gel Enantio-L1). The ^{13}C NMR spectrum (Table 4) showed 17 (2 signals were duplicated) well-defined carbon signals which were analyzed on the basis of off-resonance decoupling, considerations of substituent effect and comparison with those of feldamycin. In the spectrum determined at pH 2.0, significant upfield shifts (3~9 ppm) were observed for the carbons β to the amino functions (C-6, C-11, C-14 and C-18) in addition to carboxyl carbons (C-8 and C-12). Interpretation of these spectral data led to the complete structure for melanostatin as shown in the previous page. The absolute configuration of the diaminopropionic acid moiety (C-11) was elucidated by chemical synthesis as will be described in a later paper[12].

Biological Activity

Melanin Synthesis Inhibition in *S. bikiniensis* NRRL B-1049

Melanin Synthesis inhibition assay in *S. bikiniensis* NRRL B-1049 was performed by the procedure described in the previous paper[1]. A spore suspension of the *S. bikiniensis* was spread over modified medium ISP No. 7 (tyrosine agar medium +0.2% yeast extract) solidified in a plastic plate. After drying the agar surface, paper discs containing the test samples were placed on the surface and the plate was incubated at 28°C for 48 hours. The inhibition zone of melanin formation was measured from the back side of the plate. Inhibition zones obtained for melanostatin are shown in Table 5 along with those of four reference compounds.

Inhibition of Melanin Synthesis in B16 Melanoma Cells

Melanostatin was tested for inhibitory effect on melanin synthesis in cultured melanin-producing murine B16 melanoma cells in comparison with two related antibiotics, feldamycin and FR-900,490 and

Table 5. Inhibitory effect of melanostatin on melanin synthesis in *Streptomyces bikiniensis* NRRL B-1049.

Concentration (μg/ml)	Melanostatin	Feldamycin	FR-900,490	4-Hydroxy-anisole	Hydroquinone
250	32[a]	34	40	24	13
125	30	30	38	18	—
62.5	30	28	35	—	—

[a] Inhibition zone diameter (mm).
—: No inhibition.

Table 6. Inhibitory effect of melanostatin on melanin synthesis in B16 melanoma cells.

Compound	% Inhibition of melanin synthesis[a]								
	Concentration (μg/ml)								
	50	25	12.5	6.3	3.2	1.6	0.8	0.4	0.1
Melanostatin	62	47	35	32	9	3	0	—	—
Feldamycin	65	47	56	44	6	15	0	—	—
FR-900,490	50	47	47	47	53	15	0	—	—
Hydroquinone	—	—	—	—	—	—	—	85	69

[a] The data are mean of three experiments.
—: Not determined.

a tyrosinase inhibitor, hydroquinone. B16 cells (3×10^3 cells/ml, 3.6 ml) in EAGLE's minimum essential medium containing 10% fetal calf serum and test compounds (0.4 ml) were incubated for 6 days at 37°C in a 5% CO_2 and high humidity atomosphere. During the incubation, the culture medium was renewed once with fresh medium containing the same compound solution. On completion of incubation, cells were counted and solubilized with a mixture of 1 N NaOH and 10% DMSO (1 : 1). The amount of melanin synthesized was measured at 470 nm by a spectrophotometer. As shown in Table 6, melanostatin and feldamycin gave similar inhibition of melanin synthesis in growing B16 melanoma cells at concentrations ranging from 6.3 to 50 μg/ml. FR-900,490 was slightly more potent than the above two compounds in terms of minimum effective concentration. Hydroquinone showed markedly potent inhibition of melanin synthesis in B16 melanoma cells.

Table 7. Inhibition of mushroom tyrosinase by melanostatin.

	IC_{50} (μg/ml)
Melanostating	>200
Feldamycin	>200
FR-900,490	>200
4-Hydroxyanisole	15
Hydroquinone	1.5

Inhibition of Mushroom Tyrosinase

Inhibitory activity of melanostatin and reference compounds against mushroom tyrosinase (Sigma) was determined as reported in the previous paper[1] with the results summarized in Table 7. Melanostatin and the structurally related antibiotics did not inhibit mushroom tyrosinase at 200 μg/ml, while 4-hydroxyanisole and hydroquinone showed strong inhibition against the enzyme with IC_{50} values of 15 and 1.5 μg/ml, respectively.

Discussion

A novel melanin synthesis inhibitor, melanostatin was isolated in our screening using *S. bikiniensis* as the indicator organism. Its structure was elucidated to be 2-[(2*S*)-2-methylamino-3-phenylpropionyl]-amino-3-[(1*S*)-1-carboxy-2-(1*H*-imidazol-4-yl)ethyl]aminopropionic acid. Melanostatin differs from feldamycin and FR-900,490 at the *N*-terminal amino acid moiety where (L)-*N*-methylphenylalanine is present in melanostatin while (L)-*N*-methylhistidine and (L)-asparagine are present in feldamycin and FR-900,490, respectively. Furthermore, feldamycic acid, the common fragment of the feldamycin and FR-900,490 molecules was replaced by desmethylfeldamycic acid in melanostatin. Our experiment showed that feldamycin and FR-900,490 also have melanin synthesis inhibitory activity in *S. bikiniensis* and B16 melanoma cells[1]. Feldamycin was isolated from the metabolites of *S. ficellus* as a minor antibiotic component weakly active against Gram-positive bacteria[2]. FR-900,490 was reported to restore the decreased immune system in mammalian cells and to inhibit tumor metastasis[3]. Melanin synthesis inhibitory activity of these types of structures has previously not been reported.

Acknowledgments

The authors thank Dr. M. KOHSAKA of the Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd. for the sample of FR-900,490. They also wish to thank Prof. M. OHASHI of the University of Electro-communication for mass spectral analysis and valuable discussion. They are grateful to Dr. H. KAWAGUCHI, the consultant for their institute for his interest and encouragement and the Analytical Group for their excellent spectral analysis.

References

1) TOMITA, K.; N. ODA, M. OHBAYASHI, H. KAMEI, T. MIYAKI & T. OKI: A new screening method for melanin biosynthesis inhibitors using *Streptomyces bikiniensis*. J. Antibiotics 43: 1601~1605, 1990

2) ARGOUDELIS, A. D.; S. A. MIZSAK, L. BACZYNSKYJ & R. J. WNUK: The structure of feldamycin. J. Antibiotics 29: 1117~1119, 1976
3) TERANO, H.; Y. TSURUMI, H. SETOI, M. HASHIMOTO & M. KOHSAKA (Fujisawa Pharm.): Amino acid derivatives and production thereof. Jpn. Kokai 227554 ('86), Oct. 9, 1986
4) 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, Williams & Wilkins Co., 1974
5) 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
6) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Int. J. Syst. Bacteriol. 18: 279~392, 1968
7) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. Int. J. Syst. Bacteriol. 19: 391~512, 1969
8) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type strains of *Streptomyces*. V. Additional descriptions. Int. J. Syst. Bacteriol. 22: 265~394, 1972
9) WAKSMAN, S. A. (*Ed.*): Description of Species of *Streptomyces*. The Actinomycetes. Vol. 2. Classification, Identification and Descriptions of Genera and Species. p. 197, Williams & Wilkins Co., 1961
10) ISHIWATA, A.: High-performance liquid chromatography of amino acid enantiomers by UV detection. Abstracts of Papers of 5th Symposium on Liquid Chromatography, pp. 38~39, Tokyo, Oct. 30, 1984
11) TAKITA, T.; Y. MURAOKA, K. MAEDA & H. UMEZAWA: Selective cleavage of bleomycin. Proc. 8th Symp. on Peptide Chem., pp. 179~183, Osaka, Nov. 26~27, 1970
12) IMAE, K.; H. KAMACHI, H. YAMASHITA, T. OKITA, S. OKUYAMA, T. TSUNO, T. YAMASAKI, Y. SAWADA, M. OHBAYASHI, T. NAITO & T. OKI: Synthesis, stereochemistry, and biological properties of the depigmenting agents, melanostatin, feldamycin and analogs. J. Antibiotics 44: 76~85, 1991