M-81, A NEW PEPTIDE ANTIBIOTIC PRODUCED BY *STREPTOMYCES GRISEUS* SUBSP. *PSYCHROPHILUS* AT MODERATE TEMPERATURE

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M-81, a new water-soluble basic peptide antibiotic, was isolated from the culture filtrate of *Streptomyces griseus* subsp. *psychrophilus* AKU 2881, which produces cryomycin only at low temperatures. M-81 was produced at 20~37°C, but not below 20°C. M-81 was active against some Gram-positive bacteria. Its antimicrobial spectrum is more limited than those of cryomycin. It darkens at 208°~213°C with decomposition. The LD_{50} in mice by intraperitoneal injection is more than 300 mg/kg.

Streptomyces griseus subsp. *psychrophilus* AKU 2881 is a psychrophilic actinomycete which produces a new peptide antibiotic, cryomycin, only at low temperatures. As described in preceding papers[1,2], cryomycin is produced between 0°C and 18°C, but not above 20°C, and possesses high activity against *Bacillus subtilis* IFO 3037.

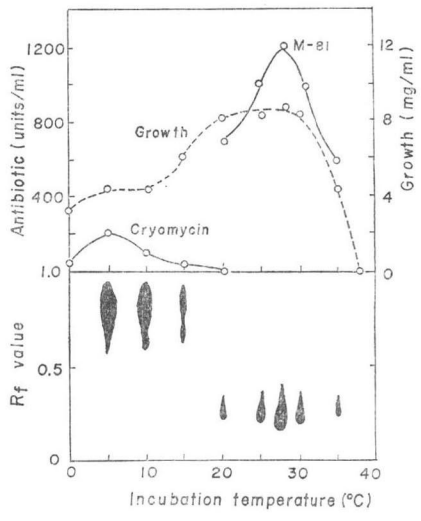
Further investigations have revealed that at moderate temperatures *Streptomyces griseus* subsp. *psychrophilus* AKU 2881 produces another antibacterial substance inactive against *B. subtilis*. This active principle was isolated, characterized as a water-soluble basic substance with a high activity against *Serratia polymuthicum* IFO 3055 and *Micrococcus lysodeikticus* IFO 3333, and named M-81. The temperature range for M-81 production is 20~37°C. It is not produced below 20°C. Its antimicrobial spectrum is narrower than that of cryomycin.

In this paper, the production, isolation and characterization of M-81 as well as its biological properties are described and compared with those of cryomycin.

Production of Antibiotic M-81

As shown in Fig. 1, *Streptomyces griseus* subsp. *psychrophilus* AKU 2881 produces two different antibiotics, cryomycin and M-81, respectively, at low and moderate temperatures. The two substances exhibit different Rf values on bioautography using butanol-acetic acid-pyridine-water (15:3:10:12) as the developing solvent and *Micrococcus lysodeikticus* IFO 3333 as the test organism. *S. griseus* subsp. *psychrophilus* grows readily on a medium of

Fig. 1. Effect of incubation temperature on growth and antibiotic formation of *Streptomyces griseus* subsp. *psychrophilus*.



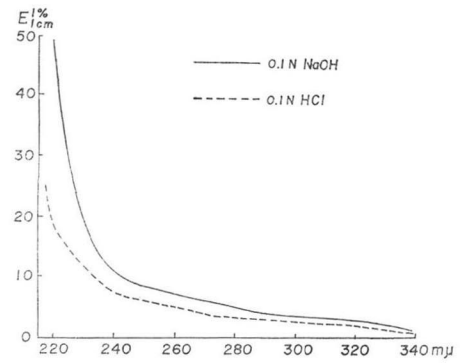
the following composition: Peptone 5 g; soluble starch 20 g; K_2HPO_4 2 g; $MgSO_4 \cdot 7H_2O$ 1 g; $FeSO_4 \cdot 7H_2O$ 0.02 g in 1,000 ml of tap water; at pH 6.5 before sterilization. The maximum yield of the antibiotic was attained after 48~72 hours. The yield of the antibiotic was markedly reduced when the organism was cultivated in a soybean-glycerol medium, which was the most suitable for the production of cryomycin.

Isolation and Purification

M-81 is found in the culture broth, but not in the mycelia. The isolation procedure is as follows: The culture broth was freed from mycelia and mixed with 1% (w/v) active carbon. The carbon was harvested, washed and eluted with 28% aqueous ammonia-water-acetone (5:45:50). The eluate was evaporated *in vacuo* to remove volatile matters, then it was passed through Dowex 1×1 ($HCOO^-$) and Dowex 1×2 (Cl^-) to remove most of the pigments and impurities. The active effluent was adsorbed on Amberlite CG-50 (H^+), followed by elution with 1N HCl-80% methanol (1:50). After being neutralized with Amberlite CG 4B (OH^-), the active eluate was condensed to a small volume and subjected to cellulose column chromatography using aqueous methanol of increasing concentration as the eluent. The active eluate was adsorbed on charcoal, followed by elution with 80% methanol.

M-81 was obtained as a pale yellow powder by concentrating the eluate *in vacuo* and lyophilizing it from the aqueous solution.

Fig. 2. Ultraviolet absorption spectrum of antibiotic M-81.



Physical and Chemical Properties

M-81 is an antibiotic powder with weakly basic properties. It darkens at 208°~213°C with decomposition. Figure 2 shows its ultraviolet absorption spectrum in water. No maxima were observed down to 210 mμ. Figure 3 shows the infrared absorption spectrum using a KBr tablet of the desolvated powder of M-81. Elementary analysis gave

Fig. 3. Infrared absorption spectrum of antibiotic M-81 (in KBr).

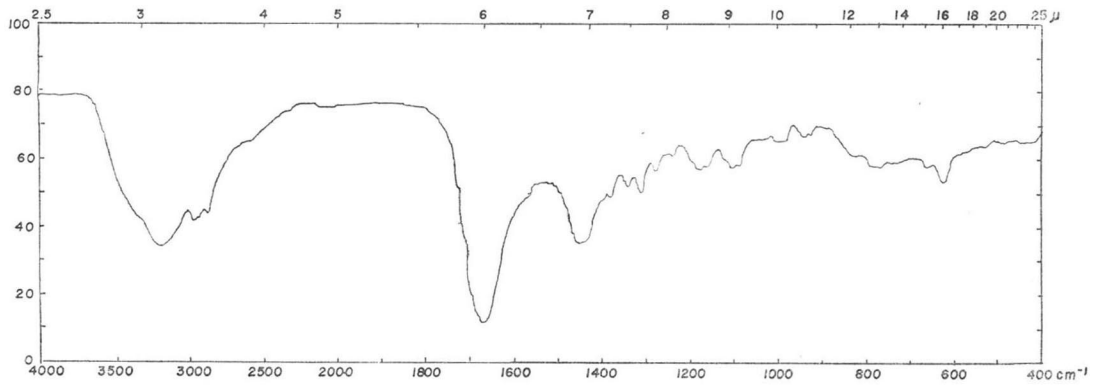


Table 1. Behavior of M-81 towards different chemical tests.

Chemical test	Result
Ninhydrin	positive
$FeCl_3$	negative
Alkaline $KMnO_4$	reduction when cold
Acidic $KMnO_4$	reduction when cold
Biuret	positive
Xanthoprotein	positive
MILLON	positive
SAKAGUCHI	positive
ADAM-KIEWICZ	positive
PAULI	positive
LIEBERMANN	negative
TOLLENS	negative
Dilute I_2 solution	decolorization in cooling
Anthrone	negative
BENEDICT	negative

Fig. 4. Salt-out paper chromatogram of M-81.

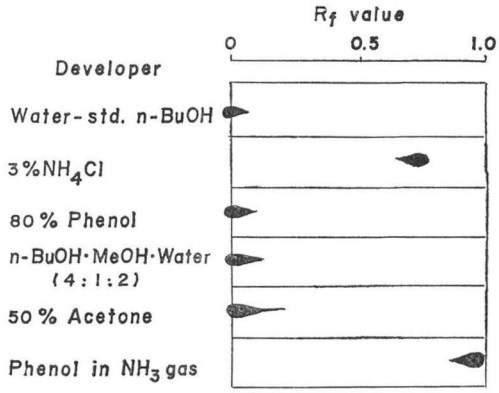


Fig. 5. Paper electrophoresis of cryomycin and M-81.

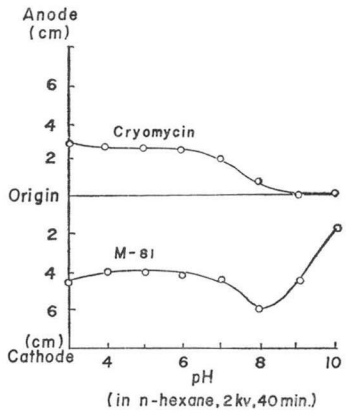
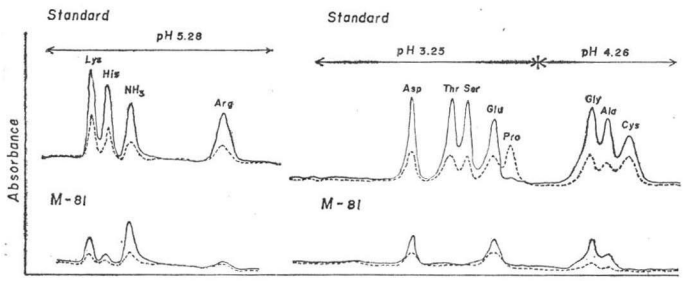


Fig. 6. Ion-exchange chromatography of antibiotic M-81 hydrolyzate.



the following composition: C 45.52, H 9.05, N 13.10.

M-81 is soluble in water, methanol and ethanol, but insoluble in other organic solvents. The chemical reactions of M-81 are given in Table 1. When M-81 was examined by paper chromatography using many solvent systems, a single active spot against *Serratia polymuthicum* IFO 3055 was observed (Fig. 4).

On paper electrophoresis at 15~20 mA and 2,000 volts for 40 minutes in each of several buffers[2], M-81 moved toward the cathode, whereas cryomycin moved toward the anode (Fig. 5).

The nitrogen content, ninhydrin reaction and the infrared absorption spectrum indicated that M-81 is a peptide antibiotic. As a result of analyzing the hydrolyzate of M-81 with an automatic amino acid analyzer, lysine, histidine, arginine, aspartic acid, glutamic acid, glycine and alanine were detected (Fig. 6). The optical configulation of these amino acids and their sequence have not yet been determined. No fatty acids were detected in the hydrolyzate.

Biological Properties

The antimicrobial spectrum of M-81 obtained by the agar dilution streak method is shown

Table 2. Antimicrobial spectrum of M-81

Microorganism	M.I.C. (mcg/ml)	Microorganism	M.I.C. (mcg/ml)
Escherichia coli IFO 3208	>100	*Streptomyces rimosus* IFO 3226	6.3
Aerobacter aerogenes IFO 3320	>100	*Saccharomyces cerevisiae* AKU* 4100	>100
Serratia polymuthicum IFO 3055	1	*Endomyces hordei* IFO 0104	3.1
Proteus vulgaris IFO 3045	>100	*Eremascus fertilis* IFO 0691	>100
Alcaligenes faecalis IFO 3160	12.5	*Schizosaccharomyces pombe* IFO 0346	>100
Flavobacterium arborescens IAM 1130	0.8	*Pichia polymorpha* IFO 0195	>100
Bacillus subtilis IFO 3037	>100	*Hansenula anomala* IFO 0118	>100
Micrococcus lysodeikticus IFO 3333	0.8	*Saccharomycodes ludwigii* IFO 0339	50
Staphylococcus aureus IFO 3061	>100	*Lipomyces lipoferus* IFO 0673	>100
Sarcina lutea IFO 3064	25	*Sporobolomyces salmonicolor* IFO 0374	>100
Corynebacterium equi IAM 1034	25	*Candida albicans* IFO 0197	>100
Arthrobacter simplex IFO 3530	25	*Trigonopsis variabilis* IFO 0671	3.1
Brevibacterium ammoniagenes IFO 12071	50	*Torula rubra* AKU 4730	>100
Bacterium cadaveris IFO 3731	>100	*Trichosporon cutaneum* IFO 0174	>100
Pseudomonas aeruginosa IFO 3080	>100	*Mucor mucedo* IFO 5776	>100
Streptococcus faecalis IFO 3181	>100	*Rhizopus oryzae* M-21	>100
Pediococcus hennevergi IFO 3884	>100	*Aspergillus oryzae* M-61	>100
Leuconostoc mesenteroides IFO 3426	>100	*Penicillium chrysogenum* IFO 4626	>100
Lactobacillus plantarum IFO 3070	>100	*Neurospora crassa* IFO 6068	>100
Propionibacterium arabinosus IAM 1714	>100	*Fusarium lini* IFO 5880	>100
Mycobacterium avium IFO 3154	>100	*Giberella fujikuroi* IFO 5268	>100
Nocardia gardneri IFO 3385	>100	*Trichophyton sulfureum* IFO 5945	>100
Streptomyces griseus IFO 3430	0.8		

* AKU: Abbreviation for Culture Collection, Faculty of Agriculture, Kyoto University, Kyoto, Japan.

in Table 2. M-81 is primarily active against Gram-positive bacteria. Its activity and spectrum are more limited than those of cryomycin.

The LD_{50} in mice of M-81 is greater than 300 mg/kg when given intraperitoneally.

Discussion

In some respects, on the basis of its properties, M-81 resembles melanomycin[3], duramycin[4,5] and amphomycin[6]. However, the nitrogen content and constitutive amino acids of melanomycin differ from those of M-81. Duramycin and amphomycin were isolated from the culture broth by extracting with butanol. Duramycin contains more kinds of amino acids, and amphomycin shows a different infrared absorption spectrum. Hence, M-81 appears to differ from known peptide antibiotics, and is recognized as a new substance. Structural details of M-81 is now being elucidated.

Incidently, it is very unusual for the same strain of microorganism to produce two quite different antibiotics at different temperature ranges. To see if the biosynthesis of M-81 and cryomycin is related, intact mycelia of *S. griseus* subsp. *psychrophilus* AKU 2881 were incubated with cryomycin at 28°C and with M-81 at 5°C. Neither the interconversion of the two antibiotics nor the degradation of either was observed[1]. Also, no increase in cryomycin production was observed by shifting the incubation temperature from 28°C to 5°C during cultivation. Cryomycin production was almost proportional to the mycelia newly developed at low temperature.[1] Thus, we suppose that cryomycin and M-81 are produced *via* independent biosynthetic

routes.

Further discussions on the biosyntheses of M-81 and cryomycin will be made in future when the interrelationships between the chemical structures of the two antibiotics will be revealed.

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