DETHYMICIN, A NOVEL IMMUNOSUPPRESSANT ISOLATED FROM AN *Amycolatopsis*

FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

MITSUHIRO UENO, MASATOMI IIJIMA, TORU MASUDA, NAOKO KINOSHITA†, HIRONOBU IINUMA†, HIROSHI NAGANAWA†, MASA HAMADA†, MASAAKI ISHIZUKA* and TOMIO TAKEUCHI

Institute for Chemotheraphy, Microbial Chemistry Research Foundation, 18-24 Aza-Motono, Miyamoto, Numazu-shi, Shizuoka 410-03, Japan
†Institute of Microbial Chemistry, Microbial Chemistry Research Foundation, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication June 29, 1992)

In the course of screening for immunomodulators inhibiting the mixed lymphocyte culture reaction (MLCR), we found a novel immunosuppressant, dethymicin in mycelium of *Amycolatopsis mediterranei* MI710-51F6. From physico-chemical properties and biological activity it is different from immunosuppressants produced by microorganisms such as cyclosporins, FK506 and rapamycin. It inhibited immune responses *in vitro* and *in vivo*, and prolonged skin allograft in rats.

In the course of screening for immunomodulators exhibiting immunosuppressive effect on mixed lymphocyte culture reaction (MLCR), we found a new immunosuppressant, dethymicin, in cultured mycelium of *Amycolatopsis mediterranei* MI710-51F6. In this paper, we report the taxonomy, fermentation, isolation and purification, physico-chemical properties and biological activities of dethymicin.

Taxonomy

The producing microorganism, strain MI710-51F6, was isolated from a soil sample collected in Hayama-machi, Kanagawa Prefecture, Japan. The cultural characteristics[1] of strain MI710-51F6 are shown in Table 1. Strain MI710-51F6 has branched vegetative hyphae with a slight tendency to zig-zag-shaped, and aerial hyphae in the form of straight or wavy. Partial fragmentation of the vegetative and aerial hyphae was observed. The aerial hyphae bore chains of ellipsoid to oblong spores. The spores are 0.3 to 0.5 by 0.9 to 1.4 μm in size with smooth surfaces. No sporangia, motile spores or synnemata were observed. This strain is not acid-fast.

Physiological characteristics[2,3] of strain MI710-51F6 are shown in Table 2.

The whole cell hydrolysate contained *meso*-2,6-diaminopimelic acids, arabinose and galactose, hence the cell wall composition is type IV and the whole-cell sugar pattern is type A[4]. The isoprenoid quinones extracted from the cells were identified as MK-9 (H_4) and MK-9 (H_2) by EI-MS. Since phosphatidylethanolamine but no phosphatidyl choline and undescribed phospholipids containing glucosamine in the cell component were found by TLC analysis[5], phospholipids were determined to be type PII. Fatty acids in methanolysates of the whole organism were analyzed by gas chromatography[6] and found major amounts of *iso*-branched 14-methylpentadecanoic acid (*i*-16), *anteiso*-branched 14-methyl-

Table 1. Cultural characteristics of strain MI710-51F6.

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigments
Sucrose-nitrate agar	Good	Shell (3ca)	Light mellon yellow (3ea)	None
Glucose-asparagine agar	Good	Shell tint ~ Shell (3ba ~ 3ca)	Bamboo (2gc)	None
Glycerol-asparagine agar (ISP-5)	Good	Light ivory ~ Light melon yellow (2ca ~ 3ea)	Light melon yellow (3ea)	None
Inorganic salts-starch agar (ISP-4)	Good	Light ivory (2ca)	Bamboo (2gc)	None
Tyrosine agar (ISP-7)	Good	Shell ~ Light melon yellow (3ca ~ 3ea)	Honey gold ~ Light melon yellow ~ Amber (2ic ~ 3ea ~ 3pc)	None
Nutrient agar	Poor	White	Honey gold (2ic)	None
Yeast extract-malt extract agar (ISP-2)	Good	Shell pink (5ba)	Light tan (3gc)	None
Oatmeal agar (ISP-3)	Good	White	Bamboo (2gc)	None
Glycerol-nitrate agar	Good	Shell ~ Light melon yellow (3ca ~ 3ea)	Light melon yellow ~ Light olive (3ea ~ $1\frac{1}{2}$ie)	None
Starch agar	Good	Shell (3ca)	Honey gold (2ic)	None

Observation after incubation at 30°C for 21 days.
Color names and numbers from Color Harmony Manual, Container Corporation of America.

Table 2. Physiological characteristics of strain MI710-51F6.

Temperature range for growth (°C)	20 ~ 37	Acid produced from	
Optimum temperature (°C)	27 ~ 30	cellobiose	Positive
Formation of melanoid pigment	Negative	*meso*-erythritol	Positive
Liquefaction of gelatin	Positive	D-glucose	Positive
Coagulation of milk	Negative	raffinose	Positive
Peptonization of milk	Positive	Utilization of	
Hydrolysis of starch	Weak	L-arabinose	+
Reduction of nitrate	Positive	D-xylose	+
Decomposition of		D-glucose	+
hypoxanthine	Positive	D-fructose	+
xanthine	Negative	rhamnose	+
Growth on 5% NaCl	+	sucrose	+
		raffinose	+
		inositol	+
		D-mannitol	+

+: Growth.

hexadecanoic acid (*a*-17) and *iso*-branched 13-methyltetradecanoic acid (*i*-15) and no mycolic acid.

Accordingly, strain MI710-51F6 is considered to be in the genus *Amycolatopsis*[7]. Among the genus *Amycolatopsis*, *Amycolatopsis mediterranei* and *Amycolatopsis orientalis* are similar to strain MI710-51F6. As shown in Table 3, strain MI710-51F6 is different from *A. orientalis* in fatty acids composition, coagulation of milk, decomposition of xanthine, acid production from raffinose and utilization of sucrose and raffinose. Strain MI710-51F6 is closely related to *A. mediterranei* except for acid production from *meso*-erythritol and utillization of raffinose. DNA relatedness among strain MI710-51F6, *A. mediterranei* IMC A-0162 (ISP 5501) and *A. orientalis* IMC A-0161 (ISP 5040) was examined by a Southern blot technique. Strain MI710-51F6 showed high homology with *A. mediterranei* qualitatively. Therefore, it was designated as *A. mediterranei* MI710-51F6.

Table 3. Comparison of strain MI710-51F6 with type strains of *Amycolatopsis mediterranei* and *Amycolatopsis orientalis*.

Characteristics	MI710-51F6	*A. mediterranei* IMC A-0162 (ISP 5501)	*A. orientalis* IMC A-0161 (ISP 5040)
Fatty acids[a]	<u>*i*-16</u>[b], *a*-17, *i*-15	<u>*i*-16</u>[b], *a*-17, *i*-15, 16:1	<u>*i*-15</u>[b], *i*-16, 16:0, 17:0, *a*-17
Color of aerial mycelium[c]	White to pale yellow orange	None to white to pale orange	White
Color of substrate mycelium[c]	Pale yellow to pale yellow orange	Dull yellow orange to light yellowish orange	Pale yellow to pale yellow orange
Coagulation of milk	Negative	Negative	Positive
Decomposition of xanthine	Negative	Negative	Positive
Acid from			
meso-erythritol	Positive	Negative	Positive
raffinose	Positive	Positive	Negative
Utilization of			
raffinose	+	−	−
sucrose	+	+	−

[a] *i*-15, *iso*-branched 13-methyltetradecanoic acid; *a*-17, *anteiso*-branched 14-methylhexadecanoic acid; 16:0, saturated hexadecanoic acid.
[b] Under line, main component.
[c] Color names from Guide to Color Standard, Nihon Shikisai Co., Ltd.
+ Growth.

Table 4. Time course of dethymicin production.

Incubation time (hours)		0	24	40	48	64	90
pH		7.0	8.2	7.7	7.2	7.1	7.0
Potency (μg/ml)[a]	sup	—	—	—	5	4	4
	myc	—	—	10	51	227	225
PMV (ml/10 ml)[b]		—	0.1	0.6	0.9	0.9	0.9

[a] Determined by HPLC using a CAPCELL PAK $5C_{18}$ (MeOH - 50 mM NH_4OAc - CH_3CN (65:30:5), 1 ml/minute, UV 235 nm).
[b] Packed mycelium volume.

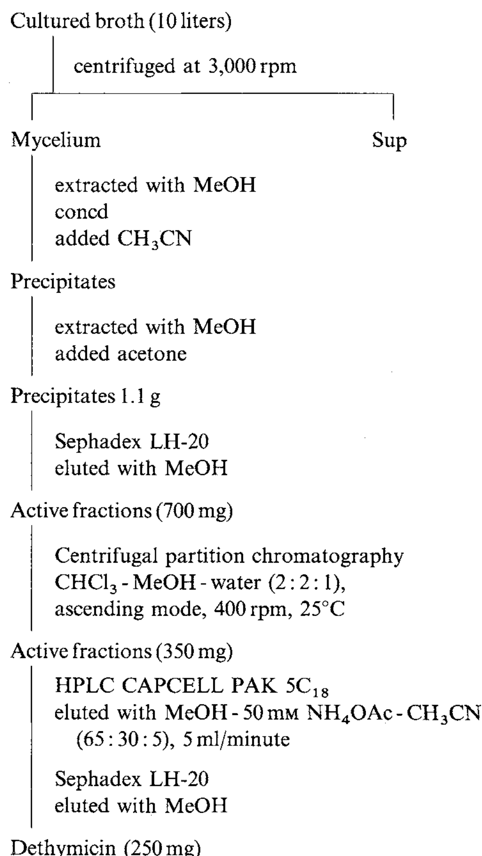
Fermentation

The medium contained glycerol 2.0%, dextrin 2.0%, Bacto soytone (Difco) 1.0%, yeast extract (Daigo Eiyo) 0.3%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2%, one drop of silicone oil (Shinetsu Kagaku) was employed for seed culture and production. The strain MI710-51F6 on an agar slant was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of the medium and cultured at 30°C for 3 days on a rotary shaker (180 rpm). Two ml of this seed culture was inoculated into 110 ml of the medium in 500-ml Erlenmeyer flasks and cultured at 28°C for 4 days on a rotary shaker (180 rpm) for production. Typical fermentation profiles for production of dethymicin in cultured broth and mycelium are shown in Table 4. The amount of dethymicin was determined by HPLC using a CAPCELL PAK $5C_{18}$ column (Shiseido) with a mobile phase of MeOH - 50 mM NH_4OAc - CH_3CN (65:30:5). As shown in Table 4, although dethymicin was found in both cultured broth and mycelium, the active principle was in the mycelium. After 64 hours cultivation, dethymicin was accumulated in broth and mycelium, 4 μg/ml and 227 μg/ml, respectively. Therefore, dethymicin was isolated from the cultured mycelium of MI710-51F6.

Isolation and Purification

The procedure for isolation and purification of dethymicin is shown in Fig. 1. The cultured broth (10 liters) was centrifuged and the mycelium was harvested. The mycelium was extracted twice with five volumes of MeOH per wet weight of mycelium. The extract was concentrated under reduced pressure to give an aqueous solution. The aqueous solution was added to the same volume of CH_3CN and was kept at 10°C to deposit precipitations. The precipitates collected by filtration was dissolved in a small amount of MeOH. To the methanolic solution, acetone was added to give a white precipitates. The precipitates collected by filtration was dissolved in MeOH and then loaded onto a Sephadex LH-20 column (4.6 × 65 cm). The activity was eluted with MeOH and concentrated under reduced pressure. The crude material (700 mg) was subjected to centrifugal partition chromatography (CPC, Sanki Engineering) previously equilibrated with the lower layer of $CHCl_3$ - MeOH - water (2 : 2 : 1) at 25°C, 400 rpm. The activity was eluted with the upper layer of $CHCl_3$ - MeOH - water (2 : 2 : 1) in the ascending mode and concentrated under reduced pressure. The resulting residue was applied to a reverse phase HPLC column (CAPCELL PAK C_{18} 20 × 250 mm, flow rate 5 ml/minute) and eluted with a mobile phase of MeOH - 50 mM NH_4OAc - CH_3CN (65 : 30 : 5). The active fractions were concentrated under reduced pressure and then loaded onto Sephadex LH-20 column. The activity was eluted with MeOH and concentrated under reduced pressure to give pure dethymicin as a white powder (250 mg).

Fig. 1. Isolation and purification of dethymicin.



Physico-chemical Properties

Physico-chemical properties of dethymicin are summarized in Table 5. It is soluble in MeOH, EtOH, DMSO, *n*-BuOH and slightly soluble in water, acetone, acetonitrile, diethyl ether, hexane. Color reactions are: positive in vanillin sulfate and negative in ninhydrin, Dragendorff, Rydon-Smith, 2,4-DNP and bromocresol green. The molecular weight and formula were determined to be $C_{71}H_{126}N_2O_{21}$ (MW 1,342) by HRFAB-MS and elemental analysis.

Biological Activities

Mixed Lymphocytes Culture Reaction (MLCR)

Spleen cells (nylon wool-passed) taken from Fisher F344 rats as the responder were mixed with spleen cells taken from WKY rats as the stimulator which had been previously incubated with 50 μg/ml of mitomycin C at 37°C for 20 minutes. The mixed cells were cultured with or without drugs in medium

Table 5. Physico-chemical properties of dethymicin.

Appearance	Colorless ~ white powder
MP	137 ~ 139°C
Molecular formula	$C_{71}H_{126}O_{21}N_2$ (EF-FAB (positive))
Mass spectrum	1,343 (M+1, FAB-MS)
Optical rotation	$[\alpha]_D^{26}$ −33.3° (*c* 0.733, MeOH)
Elemental analysis (%)	
Calcd for $C_{71}H_{126}O_{21}N_2 \cdot 4H_2O$:	C 60.23, H 9.54, O 28.25, N 1.98
Found:	C 60.33, H 9.52, O 27.48, N 2.44
UV spectrum λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$):	276 (136), 234 (137)
IR spectrum ν_{max}^{KBr} cm^{-1}:	3370, 2970, 2940, 1660, 1600, 1460, 1380, 1070
TLC[a] Rf value[b]	0.57
Rf value[c]	0.08

[a] Kieselgel $60F_{254}$ Art. 5554 (Merck).
[b] TLC solvent system (*n*-BuOH - MeOH - water, 4 : 1 : 2).
[b] TLC solvent system (2-PrOH - water, 9 : 1).

Table 6. Inhibitory effect of dethymicin on MLCR.

Dethymicin (μg/ml)	Mean ± S.D. (10^3 cpm)	Inhibition (%)
12.5	1.9±0.2	78.3***
3.13	3.1±1.0	64.0***
0.78	5.4±0.2	36.1***
0.20	6.8±1.9	19.9
None	8.5±0.3	0

*** $P<0.001$ as compared with medium control.

containing 10% fetal calf serum (FCS) at 37°C for 5 days in 5% CO_2 in air and [^{3}H]thymidine was added 16 hours before assay. MLCR was determined by measuring the incorporation of [^{3}H]thymidine into the cultured cells. Dethymicin dissolved in MeOH was diluted with RPMI1640 and added to cultures. Triplicate determinations were made. As shown in Table 6, dethymicin inhibited MLCR in a dose dependent manner at 0.78 to 12.5 μg/ml and the IC_{50} was 1.5 μg/ml.

Table 7. Inhibitory effect of dethymicin on lectin induced proliferation of splenic lymphocytes.

	Dethymicin (μg/ml)	Mean ± S.D. (× 10^2 cpm)	Inhibition (%)
None	—	14.6± 1.8	
	100	1.7± 0.4	88***
	25	6.3± 0.6	57***
	6.25	10.0± 0.4	31
Con A[a]	—	170.7±11.7	
	100	27.0± 2.5	84***
	25	74.0± 3.8	57***
	6.25	64.4±27.1	62***
	1.56	58.0±16.3	66***
	0.39	136.8±22.5	20*
	0.10	167.2± 9.4	2
LPS[b]	—	111.7± 4.8	
	100	2.5± 0.6	98***
	25	41.4± 2.1	63***
	6.25	79.7±11.4	29**
	1.56	103.8± 1.2	7

[a] Concanavalin A (Con A) in 0.5 μg/ml.
[b] Lipopolysaccharide (LPS) in 2 μg/ml.
*** $P<0.001$ as compared with medium control.
** $P<0.01$.
* $P<0.05$.

Lectin-induced Proliferation of Murine Splenic Lymphocytes

Spleen cells (1×10^6/ml) taken from CDF_1 mice were cultured in flat-bottom microplates at 37°C for 48 hours with dethymicin, and with or without lectin (0.5 μg/ml of Con A or 2 μg/ml of LPS) in 0.2 ml of RPMI1640 medium supplemented with 1% FCS. They were cultured in a fully humidified atmosphere of 5% CO_2 in air. Triplicate cultures were made in each test. Sixteen hours before assay, 0.1 μCi/well of [^{3}H]thymidine was added to each culture and the incorporation of [^{3}H]thymidine into cells was determined. As shown in Table 7, dethymicin inhibited both lectin-induced blastgenesis and showed stronger inhibition on Con A-stimulated cells than LPS-stimulated. IC_{50} values were 0.95 μg/ml and 15.0 μg/ml, respectively.

Immune Responses to Sheep Red Blood Cells (SRBC) in Mice

Antibody formation: Female CDF_1 mice (10 weeks old) were immunized on day 0 with 1×10^8 SRBC iv. Antibody formation was determined on day 4 by enumerating plaque forming cells (PFC) according to the method described previously[8]. Drug was administrated ip daily from days 1 to 3 after immunization. Dethymicin in doses of 0.78 to 50 mg/kg did not suppress antibody formation.

Delayed-type hypersensitivity response (DTH): CDF_1 mice were immunized iv with 1×10^5 SRBC. Four days later, 1×10^8 SRBC was injected subcutaneous into the left hind footpad. Twenty four hours after the elicitation, footpad thickness was measured with a caliper. Drug was injected ip daily for 3 days starting from 1 day after immunization. As

Table 8. Suppressive effect of dethymicin on DTH response to SRBC in mice.

Dethymicin[a] (mg/kg)	n	Increase of footpad thickness ($\times$0.1 mm$\pm$S.D.)	Inhibition (%)
None[b]	5	15.60$\pm$1.50	0
50	5	$-$0.10$\pm$0.00	100.6***
12.5	5	5.10$\pm$0.87	67.3**
3.13	5	16.06$\pm$1.07	$-$6.4
0.78	4	14.05$\pm$0.32	9.9

[a] Dethymicin was dissolved in DMSO-Tween 80-saline (9 : 1 : 90) and given ip daily from days 1 to 3 after immunization.
[b] Vehicle (0.25 ml) was given ip as same schedule for drug.
*** $P<0.001$ as compared with vehicle group.
** $P<0.01$.

Table 9. Prolongation of skin allograft by dethymicin.

Dethymicin[a] (mg/kg)	n	M.S.D. (days$\pm$S.D.)	T/C (%)
None[b]	11	10.27$\pm$2.28	100.0
20	8	16.38$\pm$5.29	159.4**
5	8	13.38$\pm$1.19	130.2*

[a] Dethymicin was dissolved in DMSO-Tween 80-saline (9 : 1 : 90) and given ip daily on days 1 to 9 after transplantation.
[b] Vehicle (0.25 ml) was given ip as same schedule for drug.
** $P<0.01$, * $P<0.05$ as compared with vehicle group.

Table 10. Antimicrobial activity of dethymicin.

Microorganisms	MIC (μg/ml)	Microorganisms	MIC (μg/ml)
Staphylococcus aureus FDA209P	<0.78	*Proteus vulgaris* OX19	>100
S. aureus Smith	0.1	*P. mirabilis* IFM OM-9	>100
S. aureus MS9610	0.2	*Providencia rettgeri* GN311	>100
S. aureus No. 5 (MRSA)	0.39	*P. rettgeri* GN466	>100
S. aureus No. 17 (MRSA)	0.2	*Serratia marcescens*	>100
Micrococcus luteus FDA16	<0.78	*Pseudomonas aeruginosa* A3	>100
M. luteus IFO 3333	<0.78	*P. aeruginosa* GN315	>100
M. luteus PCI 1001	1.56	*Klebsiella pneumoniae* PCI 602	>100
Bacillus anthracis	<0.78	*Mycobacterium smegmatis* ATCC 607	>100
B. subtilis NRRL B-558	3.12	*Candida tropicalis* F-1	>100
B. subtilis PCI 219	>100	*C. pseudotropicalis* F-2	>100
B. cereus ATCC 10702	<0.78	*C. albicans* 3147	>100
Corynebacterium bovis 1810	<0.78	*Candida* Yu-1200	>100
Escherichia coli NIHJ	>100	*Candida krusei* F-5	>100
E. coli K-12	>100	*Saccharomyces cerevisiae* F-7	>100
E. coli K-12 ML1629	>100	*Cryptococcus neoformans* F-10	>100
E. coli BEM11	>100	*Cochliobolus miyabeanus*	>100
E. coli BE1121	>100	*Pyricularia oryzae*	>100
E. coli BE1186	>100	*Pellicularia sasakii*	>100
Shigella dysenteriae JS11910	>100	*Xanthomonas citri*	>100
S. flexneri 4b JS11811	>100	*X. oryzae*	>100
S. sonnei JS11746	>100	*Aspergillus niger* F-16	>100
Salmonella typhi T-63	>100	*Trichophyton asteroides* 429	>100
S. enteritidis 1891	>100	*T. mentagrophytes* F-15 (833)	>100

shown in Table 8, dethymicin at 12.5 to 50 mg/kg strongly suppressed the DTH response.

Table 11. Cytotoxicity of dethymicin against tumor cells *in vitro*.

Cells	IC_{50} (μg/ml)
L-1210	14.2
EL-4	13.3
IMC carcinoma	8.5
Ehrlich carcinoma	7.7

Rat Skin Allograft

Tail skins prepared from WKY rats were transplanted to the back of Fisher F344 rats as recipients using the method previously described[9]. Drug was given ip from 1 day after the transplantation daily for 9 days. Results are shown in Table 9. Dethymicin at doses of 5~20 mg/kg significantly prolonged survival days of skin graft.

Antimicrobial Activity and Cytotoxicity

Antimicrobial activity of dethymicin was examined by serial agar dilution method using Mueller-Hinton agar (Difco) for antibacterial test which was incubated at 37°C for 18 hours and a nutrient agar containing 1% glucose for antifungal test which was incubated at 27°C for 42 hours. Minimum inhibitory concentration (MIC) value is expressed as the minimum concentration which inhibits growth of the microorganisms. As shown in Table 10, dethymicin showed antibacterial activity only against Gram-positive bacteria but not against fungi.

Cytotoxicity of dethymicin on tumor cells cultured in RPMI1640 supplemented with 10% FCS for 48 hours was determined by MTT assay. As shown in Table 11, it inhibited growth of these tumor cells at 7.7~14.2 μg/ml.

The LD_{50} of dethymicin was found to be more than 400 mg/kg ip to ICR mice.

Discussion

In order to find specific and low toxic immunosuppressants, we have searched for inhibitors of MLCR produced by microorganisms and found dethymicin in the mycelium of a strain of *Amycolatopsis*.

Immunosuppressants produced by microorganisms, cyclosporine A (Cy A)[10], FK506 (FK)[11,12] and rapamycin (RPM)[13,14] are well known. Cy A, a fungal metabolite, is a macrocyclic peptide and FK and RPM, metabolites of *Streptomyces*, are structurally related macrocyclic lactones exhibiting antifungal activities. Although dethymicin was found as an immunosuppressant produced by a microorganism, it has no antifungal activity and the physico-chemical properties (Table 5) and antimicrobial activities (Table 10) of dethymicin are entirely different from those of Cy A, FK and RPM[15,16].

On the inhibitory effect against lectin-induced proliferation of lymphocytes, the relative IC_{50} of dethymicin was stronger on Con A-induced proliferation than on that induced by LPS. It suppressed DTH response but not antibody formation to SRBC in mice. In conjunction with T cell-mediated events, dethymicin was effective in prolonging skin allograft in rats.

Results suggest that dethymicin suppressed T cell-mediated immune responses although it does not inhibit antibody formation to SRBC a T-dependent antigen in mice. Thus, it may inhibit generation of effector T cells. Dethymicin may be useful for organ transplantation and the treatment of autoimmune diseases.

The structure elucidation and mechanisms of action of dethymicin are now under study.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

References

1) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
2) GORDON, R. E.; D. A. BARNETT, J. E. HANDERHAN & C. H. PANG: *Nocardia coeliaca, Nocardia autotrophica,* and the Nocardin strain. Int. J. Syst. Bacteriol. 24: 54~63, 1974
3) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriology 56: 107~114, 1948
4) LECHEVALIER, M. P. & H. LECHEVALIER: Chemical methods as criteria for the separation of nocardiae and other actinomycetes. Biology of the Actinomycetes and Related Organism 11: 78~92, 1976
5) MINNIKIN, D. E.; P. V. PATEL, L. ALSHAMAONY & M. GOODFELLOW: Polar lipid composition in the classification of *Nocardia* and related bacteria. Int. J. Syst. Bacteriology 27: 104~117, 1977
6) SUZUKI, K. & K. KOMAGATA: Taxonomic significance of cellular fatty acid composition in some coryneform bacteria. Int. J. Syst. Bacteriology 33: 188~200, 1983
7) LECHEVALIER, M. P.; H. PRAUSER, D. P. LABEDA & J. S. RUAN: Two new genera of Nocardioform Actinomycetes: *Amycolata* gen. nov. and *Amycolatopsis* gen. nov. Int. J. Syst. Bacteriology 36: 29~37, 1986
8) ISHIZUKA, M.; H. IINUMA, T. TAKEUCHI & H. UMEZAWA: Effect of diketocoriolin B on antibody formation. J. Antibiotics 25: 320~321, 1972
9) MASUDA, T.; S. MIZUTANI, M. IIJIMA, H. ODAI, H. SUDA, M. ISHIZUKA, T. TAKEUCHI & H. UMEZAWA: Immunosuppressive activity of 15-deoxyspergualin and its effect on skin allografts in rats. J. Antibiotics 40: 1612~1618, 1987
10) BOREL, J. F.; C. FEURER, C. MAGNÉE & H. STÄHELIN: Effects of new anti-lymphocytic peptide cyclosporin A in animals. Immunology 32: 1017~1025, 1977
11) KINO, T.; H. HATANAKA, M. HASHIMOTO, M. NISHIYAMA, T. GOTO, M. OKUHARA, M. KOHSAKA, H. AOKI & H. IMANAKA: FK-506, a novel immunosuppressant isolated from a *Streptomyces*. I. Fermentation, isolation, and physico-chemical and biological characteristics. J. Antibiotics 40: 1249~1255, 1987
12) KINO, T.; H. HATANAKA, S. MIYATA, N. INAMURA, M. NISHIYAMA, T. YAJIMA, T. GOTO, M. OKUHARA, M. KOHSAKA, H. AOKI & T. OCHIAI: FK-506, a novel immunosuppressant isolated from a *Streptomyces*. II. Immunosuppressive effect of FK-506 *in vitro*. J. Antibiotics 40: 1256~1265, 1987
13) VÉZINA, C.; A. KUDELSKI & S. N. SEHGAL: Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. J. Antibiotics 28: 721~726, 1975
14) SEHGAL, S. N.; H. BAKER & C. VÉZINA: Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. J. Antibiotics 28: 727~732, 1975
15) MCALPINE, J. B.; S. J. SWANSON, M. JACKSON & D. N. WHITTERN: Revised NMR assignments for rapamycin. J. Antibiotics 44: 688~690, 1991
16) HANE, K.; M. FUJIOKA, Y. NAMIKI, T. KITAGAWA, N. KIHARA, K. SHIMATANI & T. YADUSA: Physico-chemical properties of (−)-(1R,9S,12S,13R,14S,17R,18E,21S,23S,24R,25S,27R)-17-allyl-1,14-dihydroxy-12-[(E)-2-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.$0^{4,9}$]octacos-18-ene-2,3,10,16-tetrone hydrate (FK-506). Iyakuhin Kenkyu 23: 33~43, 1992