Spicamycin, a New Differentiation Inducer of Mouse Myeloid Leukemia Cells (M1) and Human Promyelocytic Leukemia Cells (HL-60)†

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A new antibiotic was obtained from the culture broth of *Streptomyces alanosinicus 879-MT*$_3$, and the name spicamycin was given. Spicamycin had a marked effect on the induction of differentiation of human promyelocytic leukemia cells (HL-60) as well as mouse myeloid leukemia cells (M1). Its structure was elucidated by degradative studies and ^{1}H and ^{13}C NMR spectral analysis as shown in Fig. 1.

A mouse myeloid leukemia cell line (M1)[2] established from an SL strain mouse with spontaneous leukemia, and a human promyelocytic leukemia cell line (HL-60)[3] isolated from peripheral blood leukocytes of a patient with acute promyelocytic leukemia can both be induced to differentiate into mature granulocytes and macrophages by various inducers.[4] These cell lines provide good model systems for studies on mechanisms controlling myeloid cell differentiation and screening for new antitumor agents which can induce differentiation of tumor cells.[5～7]

During the course of our screening program for new differentiation inducers of myeloid leukemic cells, we isolated an active substance from the culture broth of a streptomycete, and the name spicamycin was given. It can induce differentiation of M1 cells and HL-60 cells and shows antitumor activities. This paper describes the isolation, structural determination and biological properties of spicamycin, as well as the taxonomy of its producing organism *Streptomyces alanosinicus* 879-MT$_3$.

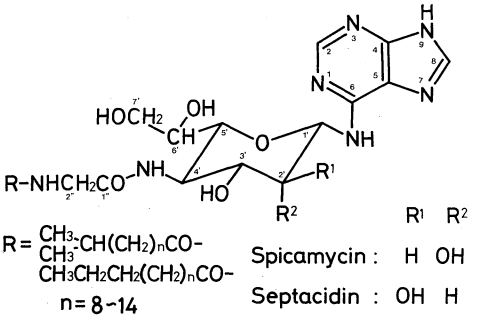

FIG. 1. Structure of Spicamycin.

Taxonomic studies of strain 879-MT$_3$

The spicamycin producing strain 879-MT$_3$ was isolated from a soil sample collected at Ichiki-cho, Kagoshima, Japan. The characterization of this organism was performed by the methods of the International Streptomyces Project (ISP)[8] and Waksman.[9]

The aerial mycelium of the strain monopodially branches on the long main stem and terminates in spirals (3～6 turns) forming long spore chains with 10 to 50 spores per chain (Fig. 2). The spores are oval (0.3～0.4×0.5～0.7 μm) with a spiny surface (Fig. 3).

* Studies on the Differentiation Inducers of Myeloid Leukemic Cells. Part VI. For Part V, see ref. 1.

The cultural and physiological properties of strain 879-MT_3 grown on various media at 37°C are shown in Tables I and II, respectively. These properties can be summarized as follows: the spore chains are spiral; the spore surface is spiny; the aerial mass color is in the red color series; the reverse side of the colony shows no distinctive pigments; the strain produces melanoid pigments, but no other soluble pigments; and all the tested sugars except L-rhamnose are utilized for growth as carbon sources.

FIG. 2. Spore Chains of Strain 879-MT_3 on Oatmeal Agar, 2 Weeks.

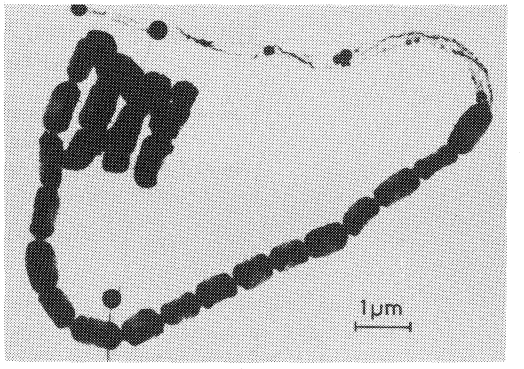

FIG. 3. Electron Micrograph of Spores of Strain 879-MT_3 on Oatmeal Agar, 2 Weeks.

TABLE I. CULTURAL PROPERTIES OF STRAIN 879-MT_3

Media	Vegetative mycelium	Aerial mycelium	Soluble pigments
Sucrose-nitrate agar (Waks. No. 1)	Moderate, brownish white to pale orange (3ca, 4ea)	Very poor, white (a)	None
Glucose-asparagine agar (Waks. No. 5)	Moderate, light yellow to dull yellow orange (2ea, 2fb, 3nc)	Moderate, velvety, red color series (4ec, 5ec)	None
Glycerol-asparagine agar (ISP No. 5)	Moderate, pale yellow (2ca)	Poor, powdery, red color series (5cb, 5ec)	None
Inorganic salts starch agar (ISP No. 4)	Moderate, pale yellow to pale yellow orange (2ca, 3nc)	Moderate, velvety, red color series (4ec, 5ec)	None
Tyrosine agar (ISP No. 7)	Poor, pale yellow (2ca)	None	None
Nutrient agar (Waks. No. 14)	Poor, brownish white (3ca)	None	None
Yeast-malt agar (ISP No. 2)	Moderate, light yellow (2ea)	Very poor, white (a)	None
Oatmeal agar (ISP. No. 3)	Moderate, light yellow (2fb)	Moderate, powdery, red color series (5eb, 5ec)	None

TABLE II. PHYSIOLOGICAL PROPERTIES OF STRAIN 879-MT_3

Temperature for growth	20~45°C
Optimum	25~37°C
Production of melanoid pigments	
Tyrosine agar	Positive
Peptone–yeast–iron agar	Positive
Tryptone–yeast agar	Negative
Hydrolysis of starch	Positive
Liquefaction of gelatin	Negative
Peptonization of milk	Positive
Coagulation of milk	Positive
Utilization of carbon sources	
Positive utilization	D-Glucose, D-fructose, D-mannose, D-xylose, L-arabinose, inositol sucrose, raffinose
Negative utilization	L-Rhamnose

On the basis of its morphological features, strain 879-MT_3 seems to belong to the genus *Streptomyces*. Among the species of *Streptomyces* described in the 8th edition of "Bergey's Manual"[10] and Shirling's ISP report,[11] the properties of strain 879-MT_3 are in good agreement with those of *S. alanosinicus*. Therefore, strain 879-MT_3 is identified as a strain of *Streptomyces alanosinicus*.

Production and isolation of spicamycin

Strain 879-MT_3 was cultivated on a rotary shaker at 37°C for four days in 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of 2.5% glucose, 1.5% soybean meal, 0.2% dry yeast and 0.4% calcium carbonate (pH 7.0).

The active material was isolated by the scheme shown in Fig. 4, and further purification was achieved by semi-preparative HPLC (Nucleosil $5C_{18}$) with methanol.

Structural determination

Spicamycin is a colorless powder soluble in methanol, *n*-butanol, pyridine and dimethylsulfoxide, but insoluble in ethyl ether, *n*-hexane, chloroform, ethyl acetate and acetone. It is also insoluble in water or 0.1 N HCl, but is soluble in 0.1 N NaOH. The melting point of spicamycin is 215~220°C with decomposition.

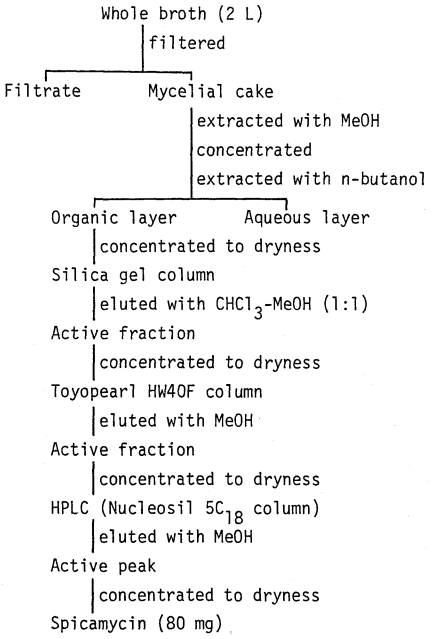

FIG. 4. Isolation and Purification of Spicamycin.

The UV spectra showed maxima at 264 nm ($E^{1\%}_{1cm}$ 257) in methanol, 273 nm ($E^{1\%}_{1cm}$ 258) in 0.01 N HCl-MeOH and 273 nm ($E^{1\%}_{1cm}$ 226) in 0.01 N NaOH–MeOH. The IR spectrum and the ^1H NMR spectrum are shown in Figs. 5 and 6, respectively. The field desorption mass spectrum revealed the molecular ion peaks at *m/z* 644 and 658 $(M+Na)^+$, thereby suggesting that spicamycin is a mixture of closely related compounds differing in the length of an alkyl chain. They are hardly separable by chromatographic procedures, the elemental analysis being as follows. Found: C 57.37, H 8.26, N 15.74, O 18.63. Calcd. for $C_{30}H_{51}N_7O_7$ (for the main component): C 57.95, H 8.27, N 15.77, O 18.01%.

On hydrolysis with 1 N HCl at 100°C for 1 hr, spicamycin was degraded into three components (Fig. 7). One was a rather insoluble acidic material, and the other two products were bases which could be separated on a Diaion HP-20 column. One of the bases was identified as adenine by direct comparison with an authentic sample. This moiety, therefore, accounts for the UV absorption of spicamycin. The second base gave characteristic

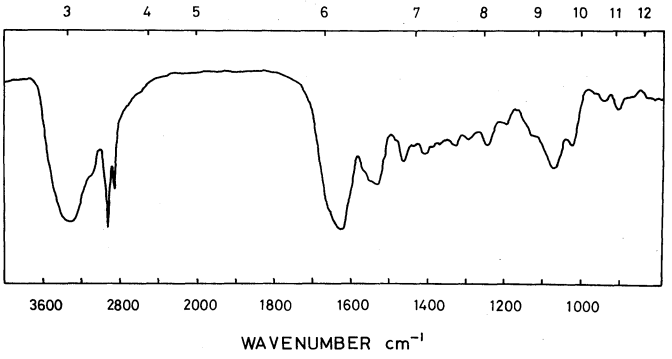

FIG. 5. IR Spectrum of Spicamycin (KBr).

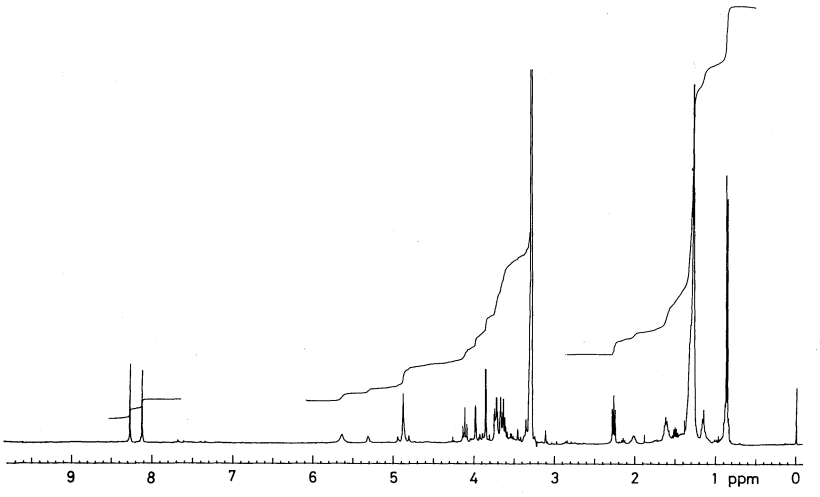

FIG. 6. 400 MHz ^{1}H NMR Spectrum of Spicamycin in Methanol-d_4.

reactions common to amino sugars.

The insoluble acidic product was hydrolyzed with 6 N HCl at 110°C for 48 hr to yield an amino acid (identified as glycine) and an acid fraction which was revealed to be a mixture of closely related fatty acids by GC-MS analysis after methylation with diazomethane (Table III). The chromatogram revealed peaks for normal and iso-type fatty acids (C_{15}–C_{18}). The major components, representing about 40% and 31% of the total, were identified as *iso*-palmitic acid and *iso*-margaric acid, respectively. Since the other three moieties of the spicamycin molecule, *i.e.*, adenine, glycine and an amino sugar were found to be homogeneous, spicamycin appears to be a family of unusual nucleoside substances containing a variety of fatty acids.

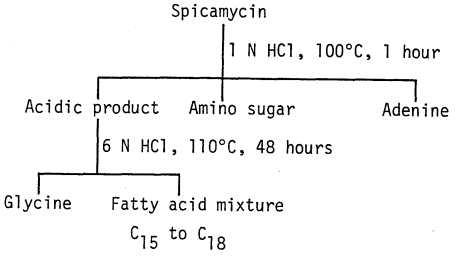

FIG. 7. Products from Hydrolysis of Spicamycin.

In the ^{13}C NMR spectrum of spicamycin in pyridine-d_5, the signals are classified into the following four groups; δ 154.0 (s), 153.0 (d), 152.6 (s), 140.6 (d) and 119.6 (s) due to the adenine residue; 80.1 (d), 79.1 (d), 74.0 (d), 73.6 (d), 71.5 (d), 64.2 (t) and 51.2 (d) ascribed

TABLE III. GC-MS ANALYSIS OF THE METHYL ESTERS OF FATTY ACIDS FROM THE HYDROLYZATE OF SPICAMYCIN

Fatty acids	t_R*	Approximate (mol%)
iso-C_{15}	0.65	6
iso-C_{16}	0.90	40
n-C_{16}	1.00	11
iso-C_{17}	1.27	31
n-C_{17}	1.33	2
iso-C_{18}	1.80	8
n-C_{18}	2.04	2

* Retention times of the methyl esters of fatty acids relative to that of methyl palmitate on a 1.5% Silicone OV-1 column at 180°C.

TABLE IV. EFFECTS OF SPICAMYCIN ON THE GROWTH AND INDUCTION OF PHAGOCYTIC ACTIVITY OF M1 CELLS

Dose (ng/ml)	Phagocytic cells (%)	Number of cells (cells/ml)
0	0	2.3×10^6
10	8	1.9×10^6
20	27	8.5×10^5
40	52	3.6×10^5
80	71	1.9×10^5
160	65	5.0×10^4
320	63	1.5×10^4

M1 cells at 2×10^5 cells/ml were incubated with various concentrations of spicamycin for 72 hr.

TABLE V. EFFECTS OF SPICAMYCIN ON THE GROWTH AND INDUCTION OF PHAGOCYTIC ACTIVITY OF HL-60 CELLS

Dose (ng/ml)	Phagocytic cells (%)	Number of cells (cells/ml)
0	3	1.2×10^6
0.6	16	9.5×10^5
2.5	51	5.9×10^5
10	58	3.4×10^5
40	72	1.7×10^5
160	71	6.0×10^4
640	61	2.5×10^4

HL-60 cells at 5×10^5 cells/ml were incubated with various concentrations of spicamycin for 48 hr.

to the aminoheptose moiety; 174.1 (s) and 43.8 (t) originating from the glycine group; and 171.7 (s), 39.2 (t), 36.3 (t), 29.6~30.1 (t), 27.6 (d), 26.0 (t) and 22.7 (q) due to the iso-type fatty acid residue.

The ^{1}H NMR spectrum of spicamycin in methanol-d_4 showed two aromatic protons (δ 8.28, 8.12) due to the adenine nucleus; an anomeric proton (δ 5.67), five oxymethine or aminomethine protons (δ 4.14, 4.00, 3.76, 3.69, 3.67) and a hydroxymethyl group (δ 3.75, 3.63) ascribed to the aminoheptose moiety; an isolated methylene group (δ 3.90, 3.85) due to the glycine moiety; and 12~13 methylene groups (δ 2.28, 1.63, 1.26~1.37, 1.16), a methine proton (δ 1.52) and two methyl protons (δ 0.88) assignable to the iso-type fatty acid moiety.

Acetylation of spicamycin with acetic anhydride in pyridine produced a tetraacetate ($C_{38}H_{59}N_7O_{11}$ for the main component). The ^{1}H NMR spectrum of the aminoheptose moiety of this substance in $CDCl_3$ displayed the following resonances: δ 6.75 (d, 4′-NH, J=8.4 Hz), 6.10 (br, H-1′), 5.53 (d, H-2′, J=3.0), 5.26 (dd, H-3′, J=10.6, 3.5), 5.15 (ddd, H-6′, J=7.2, 3.5, 2.3), 4.43 (dd, H-7′a, J=12.0, 3.5), 4.31 (dd, H-7′b, J=12.0, 7.2), 4.28 (ddd, H-4′, J=10.6, 10.5, 8.4) and 3.95 (dd, H-5′, J=10.5, 2.3). These signals indicate that the sugar moiety is a 4-aminoheptose with axial configurations for H-3′, H-4′ and H-5′, and an equatorial configuration for H-2′. Since the broad anomeric signal at 6.10 sharpened on irradiation of NH at δ 4.31, the aminoheptose moiety must be combined to the 6-amino group of the adenine residue. The axial configuration of the anomeric proton was determined by nuclear Overhauser effects (NOE) observed between H-1′ and H-3′, and between H-1′ and H-5′. These indicate that the anomeric proton has an axial configuration.

Thus, it is concluded that the structure of spicamycin is as shown in Fig. 1, with some uncertainty about the stereochemistry at the 6′ position and the absolute configuration. This structure is quite similar to that of septacidin,[12,13] an antitumor and antifungal antibiotic. Spicamycin is assumed to be the 2′-epimer of septacidin (Fig. 1).

TABLE VI. ANTITUMOR ACTIVITY OF SPICAMYCIN AGAINST P388 MOUSE LEUKEMIA

Dose (mg/kg/day)*	T/C (%)	Toxicity
0.125	129	0/6
0.25	140	0/6
0.5	153	0/6
1.0	154	0/6
2.0	154	3/6

* Treatment schedule: days 1~9 (*i.p.*).

TABLE VII. ANTIMICROBIAL ACTIVITIES OF SPICAMYCIN

Organisms	MIC (μg/ml)
Bacillus subtilis PCI 219	>100
Staphylococcus aureus FDA 209P	>100
Micrococcus luteus ATCC 9341	>100
Pseudomonas aeruginosa NCTC 10490	>100
Salmonella typhimurium IFO 12529	>100
Escherichia coli NIHJ JC-2	>100
Saccharomyces cerevisiae ATCC 9763	25
Candida albicans No. Yu 1200	>100
Candida utilis IFO 0396	25
Aspergillus fumigatus IFO 4400	>100
Penicillium chrysogenum ATCC 10002	>100
Trichophyton mentagrophytes	1.56

Biological properties

The effects of spicamycin on the induction of phagocytic activities of M1 cells and HL-60 cells are summarized in Table IV and Table V, respectively. Spicamycin at 2.5~640 ng/ml induced marked differentiation of HL-60 cells.

It showed antitumor activity against P388 leukemia in mice when intraperitoneally administered at 0.25~2.0 mg/kg/day dosages (Table VI).

The antimicrobial activities of spicamycin are shown in Table VII.

The LD_{50} in mice was approximately 40 mg/kg (i.p.).

EXPERIMENTAL

General. UV spectra were recorded using a Shimadzu UV-300 spectrophotometer. Mass spectra were measured on a JEOL DX-300 spectrometer. IR spectra were taken with a JASCO A-102 infrared spectrophotometer. NMR spectra were obtained on a JEOL FX-400 spectrometer with ^1H NMR at 400 MHz and ^{13}C NMR at 100 MHz.

For HPLC, a JASCO TRI ROTAR SR2 liquid chromatograph was used. Separations were performed on a stainless-steel column (250×8 mm i.d.) packed with Nucleosil $5C_{18}$. The column effluent was monitored at 254 nm with a JASCO UVIDEC-100-II UV detector. The mobile phase was 100% methanol and the flow rate was set at 2.0 ml/min.

For GC-MS, a Shimadzu GCMS-9000 was used. Separations were performed on a glass column (2 m×6 mm i.d.) containing 1.5% Silicone OV-1 at 180°C. Retention times of the methyl esters of fatty acids were measured relative to that of methyl palmitate.

Identifications of amino acids and bases were achieved by silica gel TLC developed with $CHCl_3$–MeOH–aq.NH_3 (2:3:1).

Isolation of spicamycin. The culture broth (2 liters) was filtered with the aid of celite, and the mycelial cake was extracted with 1 liter of methanol. After the extract had been concentrated to dryness, the residue was partitioned between 500 ml of *n*-butanol and water. The solvent layer was evaporated *in vacuo*, and the residue was subjected to silica gel column chromatography (20×4 cm i.d.). The active fraction eluted with $CHCl_3$–MeOH (1:1) was concentrated *in vacuo*, and the dried material was applied to a Toyopearl HW40F column (50×5 cm i.d.), which was developed with methanol. The active eluate was concentrated to dryness to give crude spicamycin. Further purification was achieved by HPLC on a Nucleosil $5C_{18}$ column (250×8 mm i.d.). Development of the column with methanol gave a single peak of spicamycin. After repeating this procedure, the combined fraction containing spicamycin was evaporated to dryness to give a pure sample (80 mg).

Degradation of spicamycin. Spicamycin (10 mg) was hydrolyzed with 1 N HCl at 100°C for 1 hr. The solution was cooled and extracted with ethyl acetate. The aqueous layer was subjected to a Diaion HP-20 column (20×2 cm i.d.). The effluent contained an amino sugar which reduced Fehling's reagent and gave a positive ninhydrin reaction. Development of the column with methanol gave a base fraction which was identified as adenine by direct comparison with an authentic sample. The solvent layer was evaporated *in vacuo*, and the residue was hydrolyzed with 6 N HCl at 110°C for 48 hr to yield an amino acid and a fatty acid fraction. The amino acid was identified as glycine by direct comparison with an authentic sample. The fatty acid fraction was methylated with diazomethane in ether and subjected to GC-MS analysis.

Acetylation of spicamycin. Spicamycin (10 mg) was dissolved in 2 ml of pyridine containing a slight amount of 4-dimethylaminopyridine, and 2 ml of acetic anhydride was added. Stirring for 12 hr at room temperature gave a tetraacetyl derivative of spicamycin. After concentration *in vacuo*, this material was purified by preparative

silica gel TLC developed with $CHCl_3$–MeOH (10:1) to give a pure sample of spicamycin tetraacetate ($C_{38}H_{59}N_7O_{11}$ for the main component); ^1H NMR (in CD_3Cl) δ: 8.40 (s), 7.97 (s), 6.75 (d, J=8.4 Hz), 6.37 (t, J=5.7), 6.10 (br), 5.53 (d, J=3.0), 5.26 (dd, J=10.6, 3.5), 5.15 (ddd, J=7.2, 3.5, 2.3), 4.43 (dd, J=12.0, 3.5), 4.31 (dd, J=12.0, 7.2), 4.31 (br), 4.28 (ddd, J=10.6, 10.5, 8.4), 3.98 (dd, J=16.3, 5.7), 3.95 (dd, J=10.5, 2.3), 3.90 (dd, J=16.3, 5.7), 2.27 (3H, s), 2.25 (2H, t, J=7.6), 2.04 (3H, s), 2.02 (6H, s), 1.63 (2H, m), 1.52 (m), 1.37~1.26 (18H, m), 1.16 (2H, m), 0.88 (6H, d, J=6.6).

Cells and cell culture. M1 cells were cultured in Eagle's minimum essential medium with twice the normal concentration of amino acids and vitamins supplemented with 10% heat-inactivated calf serum. HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. These cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

Assay for differentiation. Phagocytic activities were measured by using polystyrene latex particles.[2] The number of phagocytic cells were determined under a microscope, the phagocytic cells being defined as those containing five or more latex particles. Cell growth was determined from the cell number after trypan blue-stained cells had been excluded.

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REFERENCES

1) Y. Hayakawa, M. Nakagawa, H. Seto and N. Ōtake, *Agric. Biol. Chem.*, **49**, 2443 (1985).
2) Y. Ichikawa, *J. Cell. Physiol.*, **74**, 223 (1969).
3) S. J. Collins, R. C. Gallo and R. E. Gallagher, *Nature* (London), **270**, 347 (1977).
4) M. Hozumi, "Cancer Biology Reviews," Vol. 3, ed. by J. J. Marchalonis and M. G. Hanna, Jr., Marcel Dekker, Inc., New York, 1982, p. 153.
5) Y. Hayakawa, M. Nakagawa, T. Ando, A. Shimazu, H. Seto and N. Ōtake, *J. Antibiot.*, **35**, 1252 (1982).
6) A. Kawashima, M. Nakagawa, Y. Hayakawa, H. Kawai, H. Seto and N. Ōtake, *J. Antibiot.*, **36**, 173 (1983).
7) Y. Hayakawa, M. Nakagawa, H. Kawai, K. Tanabe, H. Nakayama, A. Shimazu, H. Seto and N. Ōtake, *J. Antibiot.*, **36**, 934 (1983).
8) E. B. Shirling and D. Gottlieb, *Intern. J. Syst. Bacteriol.*, **16**, 313 (1966).
9) S. A. Waksman, "The Actinomycetes," Vol. 2, The Williams and Wilkins Co., Baltimore, 1961.
10) R. E. Bauchman and N. E. Gibbson (ed.), "Bergey's Manual of Determinative Bacteriology," 8th Ed., The Williams and Wilkins Co., Baltimore, 1974.
11) E. B. Shirling and D. Gottlieb, *Intern. J. Syst. Bacteriol.*, **18**, 69 (1968); **18**, 279 (1968); **19**, 391 (1969); **22**, 265 (1972).
12) J. D. Dutcher, M. H. von Saltza and F. E. Pansy, *Antimicrob. Agents Chemother.*, 83 (1963).
13) H. Agahigian, G. D. Vickers, M. H. von Saltza, J. Reid, A. I. Cohen and H. Gauthier, *J. Org. Chem.*, **30**, 1085 (1965).