# MACROLIDE ANTIBIOTICS M-4365 PRODUCED BY *MICROMONOSPORA*

## I. TAXONOMY, PRODUCTION, ISOLATION, CHARACTERIZATION AND PROPERTIES

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(Received for publication March 18, 1977)

A series of basic 16-membered macrolide antibiotics, M-4365 $A_1$, $A_2$, $A_3$, $G_1$, $G_2$ and $G_3$, were isolated from the culture broth of strain MCRL 0940 which is assigned to be a new species of *Micromonospora* and for which the name *Micromonospora capillata* sp. nov. is proposed. Among these antibiotics, M-4365 $A_2$ and $G_2$ showed strong inhibitory activity against Gram-positive and Gram-negative bacteria.

Hitherto, megalomicin[1], rosamicin[2], antibiotic XK-41[3], juvenimicin[4] and erythromycin[5] have been reported as the basic macrolide antibiotics produced by *Micromonospora*. In screening for new antibiotics produced by *Micromonospora*, a complex of basic 16-membered macrolide antibiotics was obtained from the culture broth of strain MCRL 0940. This complex, designated M-4365, consisted of at least six components designated M-4365 $A_1$, $A_2$, $A_3$, $G_1$, $G_2$ and $G_3$. The antibiotics-producing strain is considered as a new species of *Micromonospora* based on its micromorphological, cultural and physiological characteristics, and the name *Micromonospora capillata* sp. nov. is proposed for this microorganism. Among six components, M-4365 $A_2$ and $G_2$ showed strong activity against Gram-positive and Gram-negative bacteria. As a result of structure elucidation which will be dealt within the succeeding paper[6], M-4365 $A_1$, $G_1$ and $G_2$ were concluded to be new. However, M-4365 $A_2$, $A_3$ and $G_3$ were identified with rosamicin (juvenimicin $A_3$), juvenimicins $A_4$ and $B_1$, respectively. This paper deals with the taxonomy of the producing strain, isolation and purification of the M-4365 components and their physicochemical and biological properties.

### Characteristics of Strain MCRL 0940

Strain MCRL 0940 was isolated from a soil sample collected in Kiryu City, Gumma, Japan. The taxonomic study was generally carried out by the methods adopted by the International Streptomyces Project (ISP)[7] using the media recommended by SHIRLING-GOTTLIEB[7] and WAKSMAN[8]. Colors were described according to the color names and hue numbers of the Color Harmony Manual (4th edition)[9].

#### Morphological Characteristics

Strain MCRL 0940 grew better on organic media than synthetic media. On organic agar media, vegetative mycelia raised, granulated and developed into the medium. At the top of a short sporophore it bore a single brownish black to black spore which, at maturity, is spherical to oval (0.7~0.8 micron). Spore-surface showed a warty-like structure (Plate 1). Rudimentary and retarded aerial mycelia were formed as white patches on inorganic salts starch agar (Plate 2).

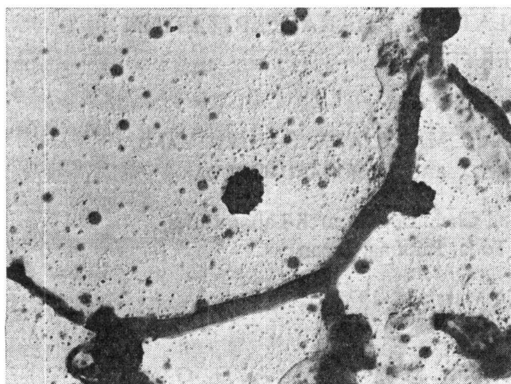

Plate 1. Electronmicrograph of *Micromonospora capillata*. (Yeast extract-malt extract agar, ×10,000)

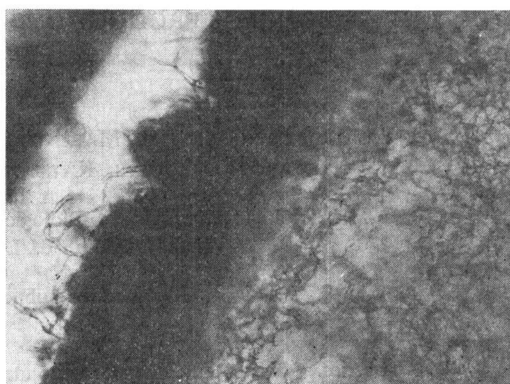

Plate 2. Photomicrograph of *Micromonospora capillata*. (Inorganic salts starch agar)

## Cultural Characteristics

The cultural characteristics of strain MCRL 0940 are summarized in Table 1.

## Physiological Properties

Tests for starch hydrolysis (ISP medium No. 4), nitrate reduction (Difco nitrate broth), milk coagulation and peptonization (Difco 10% skimmed milk), hydrogen sulfide (Difco peptone iron agar containing 0.1% yeast extract) and cellulase production (CZAPEK's solution with a strip of filter paper as the sole carbon source) were positive, but a test for tyrosinase (ISP medium No. 7 and Waksman medium No. 42) was negative. Strain MCRL 0940 was aerobic and showed good growth at 37°C in a pH range of 7.0~8.0. The strain could grow even at pH 5.0 or 9.0 at 18°~45°C.

## Utilization of Carbon Sources

Utilization of carbon sources was examined using ISP medium No. 9 as a basal medium. L-Arabinose, D-glucose, maltose, D-mannose, L-rhamnose, starch and sucrose were well, and D-fructose, D-galactose, D-melibiose and D-raffinose were poorly utilized, while dulcitol, glycerol, inositol, inulin, lactose, D-mannitol, salicin and D-sorbitol were not utilized.

Table 1. Cultural characteristics of strain MCRL 0940

| Medium | Growth | Reverse | Spore | Soluble pigment |
|---|---|---|---|---|
| Sucrose nitrate agar (Waksman medium No. 1, 27°C) | moderate, camel (3 ie) | light tan (3 gc) | lamp black (p) | covert tan (2 ge) |
| Glucose asparagine agar (Waksman medium No. 2, 27°C) | moderate, light tan (3 gc) | apricot (4 ga) | chocolate brown (4 pn) | none |
| Glycerol asparagine agar (ISP medium No. 5, 27°C) | poor, biscuit (2 ec) | biscuit (2 ec) | none | none |
| Inorganic salts starch agar (ISP medium No. 4, 27°C) | moderate, bisque (3 ec) | light tan (3 gc) | lamp black (P) with oyster white (b) aerial mycelia | none |
| Tyrosine agar (ISP medium No. 7, 27°C) | poor, light amber (3 ic) | light tan (3 gc) | sepia brown (3 pn) | none |
| Nutrient agar (Waksman medium No. 14, 27°C) | moderate, nude tan (4 gc) | bisque (4 ec) | lamp black (p) | baby pink (7 ca) |
| Yeast extract-malt extract agar (ISP medium No. 2, 27°C) | good, light tan (3 gc) | light tan (3 gc) | lamp black (p) | beige (3 ge), slightly |
| Oat meal agar (ISP medium No. 3, 27°C) | moderate, melon yellow (3 ga) | | lamp black (p) | none |

Strains which are mesophilic, bear a single spore at the top of a sporophore and do not form a true aerial mycelium are classified as *Micromonospora*. According to the criteria of *Micromonospora* taxonomy by LUEDEMANN, *et al.*[10], strain MCRL 0940 belongs to a type B or C, based on its morphological features. *Micromonospora carbonacea* var. *carbonacea* LUEDEMANN and BRODSKY 1965[10] and *Micromonospora carbonacea* var. *aurantica* LUEDEMANN and BRODSKY[10] belong to this type. However, strain MCRL 0940 is differentiated from these strains in colors of vegetative growth and soluble pigment, ability of forming aerial mycelia and utilization patterns of lactose and rhamnose. Further, *Micromonospora echinospora* and its variety[11] are known to form a spiny or warty-like structure on the surface of the spore. However, their color of vegetative growth, formation of aerial mycelia and utilization pattern of carbon sources differ from those of strain MCRL 0940. Furthermore, strain MCRL 0940 was clearly distinguished from a rosamicin-producing *Micromonospora rosaria* WAGMAN, WAITZ, MARQUEZ, MURAWSKI, ODEN, TESTA and WEINSTEIN, 1972[2], because the latter formed a wine red soluble pigment, showed positive hydrolysis of gelatin, did not form rudimentary aerial mycelia, and utilized mannitol and lactose but not raffinose. As a result, strain MCRL 0940 is considered to be a new species by the phenomenon that the strain formed hairly-like rudimentary aerial mycelia, and thus the name *Micromonospora capillata* FURUMAI and OKUDA sp. nov., is proposed. The type strain (MCRL 0940) has been deposited in the culture collections of the Fermentation Research Institute, Chiba, Japan, under the accession number of FERM-P 2598.

## Fermentation

Production of M-4365 complex was carried out in a jar fermentor. Total potency in a broth was measured by cup-plate method using *Bacillus subtilis* ATCC 6633 as a test organism and expressed as M-4365 $A_2$ which was used as a standard material for a bioassay. It was experienced that total potency and a production ratio of each component during fermentation differ markedly depending upon the medium and temperature adopted. For example, cultivation at 27°C in the A-medium listed in Table 2 gave predominantly A group components (rich in M-4365 $A_2$), while in the G-medium at 37°C all components were simultaneously produced in the broth. In general, total potency was 5 to 10 times less under the latter conditions. In the present experiment, fermentation at 37°C in the G-medium was preferred so as to acquire all components of M-4365. A seed culture was prepared by inoculating spores to four 500-ml Erlenmeyer flasks provided with 100 ml of the G-medium and cultivating for 60 hours at 37°C on a rotatory shaker. Then, 450 ml of a seed culture thus obtained was inoculated to a 30-liter jar fermentor provided with 15 liters of the sterilized G-medium and cultivated under the following conditions: temperature, 37°C; aeration 8 liters per min.; agitation, 200 r.p.m.; internal pressure, 0.5 kg per cm$^2$. Thus, production of 15 mcg per ml of M-4365 complex was attained after about 40 to 60 hours.

Table 2. Production media of M-4365

| A medium | | G medium | |
|---|---|---|---|
| Sucrose | 2.0% | Soluble starch | 2.4% |
| Glucose | 1.5% | Glucose | 0.1% |
| Beef extract | 0.75% | Beef extract | 0.3% |
| Yeast extract | 0.1% | Yeast extract | 0.5% |
| Peptone | 0.5% | Triptone | 0.5% |
| $MgSO_4 \cdot 7H_2O$ | 0.2% | $CaCO_3$ | 0.2% |
| $K_2HPO_4$ | 0.1% | | |

## Isolation

The isolation of M-4365 complex was carried out as shown in Chart 1. Thus, 4.2 g of the crude powder was obtained from 50 liters of the fermentation broth. A ratio of each component in the crude powder was determined by

Chart 1. Isolation and purification of M-4365 components

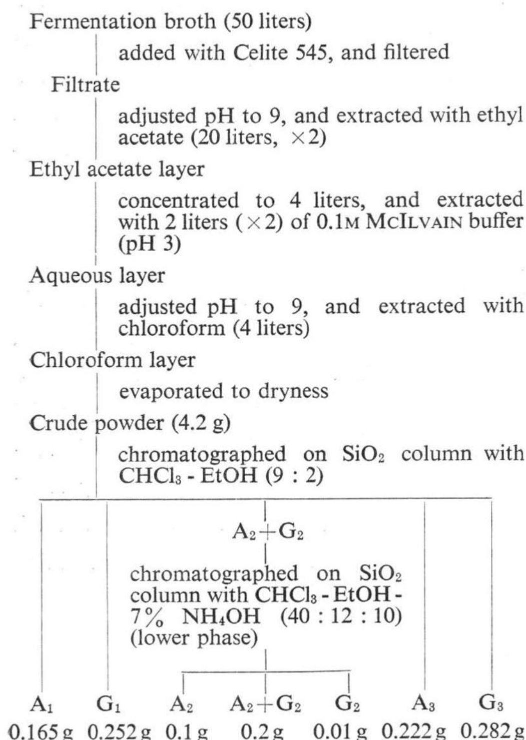


Fig. 1. U.V. Spectrophotometric survey of M-4365 complex.

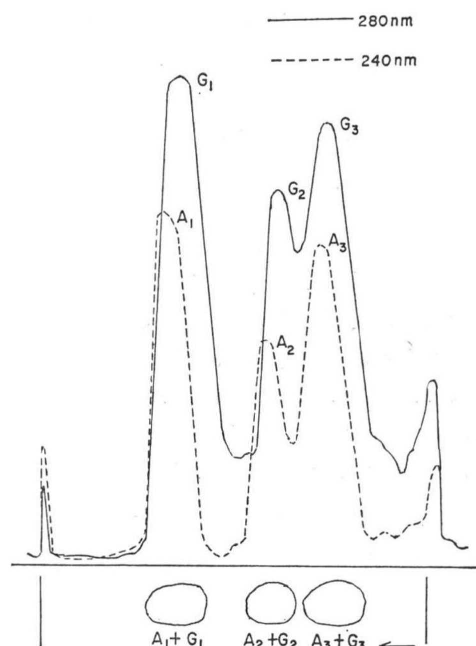


Table 3. Physicochemical properties of M-4365 components

| | M-4365 $A_1$ | M-4365 $A_2$ | M-4365 $A_3$ | M-4365 $G_1$ | M-4365 $G_2$ | M-4365 $G_3$ |
|---|---|---|---|---|---|---|
| m.p. | 97~101°C | 119~122°C | 101~105°C | 86~90°C | 106~110°C | 102~104°C |
| Formula | $C_{31}H_{53}NO_8$ | $C_{31}H_{51}NO_9$ | $C_{31}H_{53}NO_9$ | $C_{31}H_{53}NO_7$ | $C_{31}H_{51}NO_8$ | $C_{31}H_{53}NO_8$ |
| M.W. ($M^+$) | 567 | 581 | 583 | 551 | 565 | 567 |
| Elementary analysis | Calcd. Found. | Calcd. Found. | Calcd. Found. | Calcd. Found. | Calcd. Found. | Calcd. Found. |
| C: | 65.58 65.64 | 64.00 65.05 | 63.78 63.55 | 67.48 67.38 | 65.81 65.56 | 65.58 65.33 |
| H: | 9.41 9.24 | 8.84 8.93 | 9.15 9.31 | 9.68 9.40 | 9.09 9.11 | 9.41 9.42 |
| N: | 2.47 2.29 | 2.41 2.35 | 2.40 2.51 | 2.54 2.68 | 2.48 2.50 | 2.47 2.40 |
| $[\alpha]_D^{26}$ (MeOH) | −14° (c 0.2) | −35° (c 0.67) | −20° (c 0.3) | −5° (c 0.1) | −16° (c 0.33) | −18° (c 0.15) |
| U.V. $\lambda_{max}^{EtOH}$ | 240 nm (log ε 4.12) | 240 nm (log ε 4.12) | 240 nm (log ε 4.09) | 283 nm (log ε 4.17) | 283 nm (log ε 4.19) | 283 nm (log ε 4.21) |
| Rf value* | 0.97 | 0.74 | 0.65 | 0.75 | 0.68 | 0.5 |
| I.R. $\nu_{max}^{nujol}$ ($cm^{-1}$) | 3500, 1725, 1690, 1620, 1310, 1180, 1110, 1075, 1045, 980 | 3500, 2710, 1720, 1690, 1615, 1310, 1180, 1105, 1065, 1040, 980 | 3450, 1730, 1690, 1620, 1315, 1280, 1180, 1110, 1070, 1040, 980 | 3500, 1715, 1685, 1625, 1595, 1315, 1290, 1180, 1110, 1050, 980 | 3460, 2720, 1740, 1725, 1680, 1630, 1585, 1315, 1180, 1110, 1075, 1045, 985 | 3450, 1720, 1680, 1630, 1590, 1315, 1290, 1180, 1110, 1070, 1045, 980 |

* Silica gel (Toyo roshi, Spot-film), Solvent system: $CHCl_3$ - MeOH - 7% $NH_4OH$ (40 : 12 : 10) lower phase

U.V. spectrophotometric measurement (U.V. spectrophotometer, Hitachi MPF-2A) of a thin-layer chromatogram obtained by developing a plate (silica gel $GF_{254}$, Merck) with the lower phase of a mixture of $CHCl_3$-MeOH-7% $NH_4OH$ (10:3:5, v/v). The A group components were sensitive to 240 nm and G group components were so to 280 nm (cf. U.V. data in Table 3). Therefore, by surveying a plate with 240 and 280 nm, each component on a plate was independently detected as shown in Fig. 1. By calculating the area dimentions shown by each component, the above crude powder is roughly estimated to consist of M-4365 $A_1$, $A_2$, $A_3$, $G_1$, $G_2$ and $G_3$ in a ratio of 20: 10: 20: 40: 15: 30. Then, 4.2 g of the crude powder was submitted to silica gel (Merck) column chromatography using the solvent system of $CHCl_3$ - EtOH (9: 2). Each eluate was monitored by TLC on a silica gel $GF_{254}$ plate with the solvent system of $CHCl_3$ - MeOH - 7% $NH_4OH$ (40: 12: 10, lower phase), detection being made by spraying with 40% $H_2SO_4$ followed by heating. Thus, six components could be obtained respectively as a white amorphous powder. Compared to the ratio in the crude powder, recovery of M-4365 $G_2$ was very low. This could be due to the unsuccessful separation of the $A_2$ and $G_2$ components.

## Physicochemical Properties

Each component obtained as a white amorphous powder is basic in nature and soluble in lower alcohols, lower alkyl acetates, chloroform, acetone and benzene, but hardly soluble or insoluble in water, petroleum ether, *n*-hexane and cyclohexane. These components are positive to $Br_2$ and $KMnO_4$ tests, but negative to carbomycin and erythromycin tests, ninhydrin and ferric chloride color reactions.

Table 4. Antimicrobial and antimycoplasma spectra of M-4365 components

| Test organisms | M* | M.I.C. (mcg/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | $A_1$ | $A_2$ | $A_3$ | $G_1$ | $G_2$ | $G_3$ |
| *Staphylococcus aureus* 209P JC-1 | I | 3.12 | 0.195 | 3.12 | 3.12 | 0.195 | 3.12 |
| *S. aureus* Smith | I | 3.12 | 0.195 | 1.56 | 3.12 | 0.195 | 3.12 |
| *S. aureus* T-88** | I | >100 | >100 | >100 | >100 | >100 | >100 |
| *S. aureus* B-56 | I | 1.56 | 0.195 | 3.12 | 3.12 | 0.39 | 6.25 |
| *S. epidermidis* 10131 | I | 3.12 | 0.195 | 0.78 | 3.12 | 0.195 | 3.12 |
| *S. epidermidis* Kawamura | I | 3.12 | 0.195 | 3.12 | 3.12 | 0.195 | 3.12 |
| *Sarcina lutea* ATCC 9341 | I | 3.12 | 0.195 | 0.78 | 3.12 | 0.195 | 0.39 |
| *Streptococcus faecalis* | I | 12.5 | 0.39 | 25 | 25 | 0.78 | 12.5 |
| *Bacillus subtilis* ATCC 6633 | I | 0.78 | 0.098 | 1.56 | 1.56 | 0.195 | 0.78 |
| *Escherichia coli* NIHJ JC-2 | I | 100 | 12.5 | 100 | 100 | 12.5 | 50 |
| *E. coli* K-12 | I | 25 | 6.25 | 50 | 50 | 6.25 | 25 |
| *E. coli* FE-216 | I | 100 | 12.5 | 100 | 100 | 12.5 | 100 |
| *Salmonella typhimurium* | I | 100 | 25 | 100 | 100 | 12.5 | 100 |
| *Klebsiella pneumoniae* | I | 0.39 | 0.78 | 0.78 | 0.39 | 0.78 | 0.78 |
| *Pseudomonas aeruginosa* $A_3$ | I | 100 | 12.5 | 100 | 100 | 12.5 | 100 |
| *P. aeruginosa* 87 | I | 100 | 50 | 100 | 100 | 12.5 | 100 |
| *Proteus vulgaris* | I | 100 | 12.5 | 50 | 100 | 6.25 | 100 |
| *Mycoplasma pneumoniae* Mac | II | 0.31 | 0.05 | 0.31 | 0.01 | 0.005 | 0.31 |
| *M. gallisepticum* KP-13 | II | 0.31 | 0.05 | 0.16 | 0.01 | 0.005 | 0.62 |
| *M. gallisepticum* PG-31 | II | 0.62 | 0.05 | 0.62 | 0.01 | 0.005 | 0.62 |
| *M. pulmonis* mA | II | 0.62 | 0.05 | 0.62 | 0.01 | 0.05 | 0.62 |
| *Acholeplasma laidlawii* | II | 5 | 0.39 | 5 | 0.31 | 0.31 | 5 |

* Medium I: Heart infusion agar (Eiken) Medium II: PPLO broth (Difco)+10% horse serum

** Macrolide resistant strain

Table 5. Antimicrobial spectra of M-4365 $A_2$ and $G_2$, erythromycin (EM) and leucomycin $A_3$ (LM-$A_3$)

| Test organisms | M* | M.I.C. (mcg/ml) | | | |
|---|---|---|---|---|---|
| | | $A_2$ | $G_2$ | EM | LM-$A_3$ |
| *Staphylococcus aureus* 209P JC-1 | I | 0.19 | 0.19 | 0.1 | 0.39 |
| *S. aureus* Smith | I | 0.19 | 0.19 | 0.1 | 0.19 |
| *S. aureus* 199 R** | I | >100 | >100 | >100 | >100 |
| *S. epidermidis* 10131 | I | 0.19 | 0.1 | 0.05 | 0.19 |
| *S. epidermidis* Kawamura | I | 0.19 | 0.19 | 0.1 | 0.19 |
| *Streptococcus faecalis* | I | 0.19 | 0.39 | 0.1 | 0.39 |
| *Bacillus subtilis* ATCC 6633 | I | 0.19 | 0.05 | 0.05 | 0.19 |
| *Corynebacterium diphtheriae* P.W. 8 | II | 0.1 | 0.05 | 0.1 | 0.19 |
| *Mycobacterium smegmatis* ATCC 607 | III | 100 | 100 | 100 | 100 |
| *Neisseria gonorrhoeae* | II | 0.05 | 0.05 | 0.39 | 0.39 |
| *N. meningitidis* | II | 0.2 | 0.1 | 0.39 | 3.12 |
| *Escherichia coli* NIHJ JC-2 | I | 12.5 | 12.5 | 50 | 100 |
| *E. coli* K-12 | I | 6.25 | 6.25 | 100 | 100 |
| *Salmonella typhimurium* | I | 12.5 | 6.25 | 100 | 100 |
| *Klebsiella pneumoniae* | I | 3.12 | 3.12 | 50 | 100 |
| *Proteus mirabilis* 1698 | I | 50 | 6.25 | 100 | 100 |
| *P. morganii* KU-127 | I | 25 | 100 | 100 | 100 |
| *Pseudomonas aeruginosa* $A_3$ | I | 12.5 | 12.5 | 50 | 100 |

* Medium I: Heart infusion agar (Eiken)
Medium II: Heart infusion agar (Eiken)+10% horse blood
Medium III: DUBOS agar
** Streptomycin, tetracycline, penicillin and macrolide resistant strain

The other physicochemical properties of individual components are listed in Table 3.

As will be reported in the succeeding paper, M-4365 $G_2$ could be obtained in a great quantities by chemical treatment of M-4365 $A_2$.

## Biological Properties

The antimicrobial and antimycoplasma activities of M-4365 components are shown in Table 4. In Table 5, the antimicrobial activities of M-4365 $A_2$ and $G_2$ and those of erythromycin and leucomycin $A_3$ for comparison are illustrated. As shown in these tables, all components showed an inhibitory activity against the majority of Gram-positive bacteria, *Klebsiella pneumoniae* and *Mycoplasma* spp. However, cross resistance was observed with other macrolide antibiotics. Details of the biological properties of M-4365 $A_2$ and $G_2$ will be reported elsewhere.

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