# METHYLENOLACTOCIN, A NOVEL ANTITUMOR ANTIBIOTIC FROM *PENICILLIUM* SP.

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A novel antitumor antibiotic, methylenolactocin, was isolated from the culture filtrate of a new isolate of fungus identified as *Penicillium* sp. The fermentation yield reached about 100 mg per liter of the broth. Methylenolactocin has the molecular formula of $C_{11}H_{16}O_4$ and possess an exomethylene lactone structure. Its structure was determined to be 3-carboxy-2-methylene-4-nonanolide by spectroscopic data. It is active against some Gram-positive bacteria and it prolongs the life span of mice inoculated with Ehrlich carcinoma.

During the course of screening[1] for potential antitumor antibiotics from molds by use of a *Bacillus subtilis* IFO 12210 as a test organism, an active substance has been isolated from the culture filtrate of a *Penicillium* sp. strain No. 24-4 which was isolated from a soil sample picked up in a farmyard of Takatsuki-city, Osaka Prefecture, Japan. The assay for antitumor antibiotics was based on Michael addition reaction and the antibiotic whose activity was reversed by glutathione was chosen. The chemical investigations revealed that the antibiotic was 3-carboxy-2-methylene-4-nonanolide. The new substance was designated methylenolactocin indicating the presence of a methylene and a lactone in the chemical structure. The present paper deals with the taxonomy of the producing organism, fermentation, isolation, structure determination and biological characteristics of methylenolactocin.

## Materials and Methods

### Taxonomy of the Strain

Morphological and cultural studies were carried out using the following media.

CZAPEK's agar; $NaNO_3$ 3 g, $K_2HPO_4$ 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, sucrose 30 g, agar 15 g, distilled water 1,000 ml.

Malt extract agar; malt extract 20 g, Polypepton 1 g, glucose 20 g, agar 15 g, distilled water 1,000 ml.

Potato - dextrose agar (PDA); 500 g of sliced potato in about 600 ml of water was warmed at 60°C for 1 hour, filtered and added glucose 20 g, agar 15 g and distilled water to 1,000 ml.

Sabouraud agar; glucose 40 g, Polypepton 10 g, agar 15 g, distilled water 1,000 ml.

### Fermentation Procedure

A loopful of conidia from *Penicillium* sp. strain No. 24-4 was inoculated in 100 ml of the medium in a 500-ml Erlenmeyer flask. The medium composition was glucose 30 g, soybean meal 2 g, yeast extract 0.5 g, $KH_2PO_4$ 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, NaCl 0.5 g, $CaCl_2 \cdot 2H_2O$ 0.5 g, $FeCl_3 \cdot 6H_2O$ 2.0 mg and $ZnSO_4 \cdot 7H_2O$ 3.0 mg in 1,000 ml distilled water and it was adjusted to pH 5.5 before sterilization. Fermentation was carried out at 30°C for 5 days on a shaking machine. Scale up fermentation was

carried out in 2,000 ml of the same medium in a 5,000-ml Erlenmeyer flask inoculated with 50 ml of seed culture at 30°C on a rotary shaker at 167 rpm for 5 days. Antimicrobial activity was assayed by paper-disc agar diffusion method using *B. subtilis* IFO 12210 as a test organism.

Physico-chemical Measurements

The mp was determined on a microscope hot plate and uncorrected. The optical rotation was recorded on a Jasco DIP-SL automatic polarimeter. The IR spectrum was obtained with a Jasco IRA-2. The UV spectrum was measured on a Shimadzu double-beam spectrometer UV-180. The low and high resolution mass spectra were obtained on a Hitachi RMU-6M and a Jeol JMS D-300 mass spectrometer, respectively. The $^{1}H$ and $^{13}C$ NMR spectra were measured on a Jeol FX-100 spectrometer.

Antimicrobial Assay

The MIC of methylenolactocin was determined by the agar dilution method using bouillon agar for bacteria and Sabouraud agar for fungi and yeasts. Observation was made after 18 hours for bacteria and 48 hours for fungi and yeasts at 30°C following inoculation of test organisms.

Antitumor Activity

To determine the antitumor effect of methylenolactocin, $2\times10^{6}$ of Ehrlich carcinoma cells were inoculated intraperitoneally into each ICR mouse (female, 5 weeks old) and 0.05 ml, 0.1 ml and 0.2 ml of an ethanol solution of methylenolactocin was injected into the mice intraperitoneally once every day for 10 days, starting on the day of the tumor cells inoculation. A drug solution was made by mixing one part of ethanol solution of methylenolactocin (5 mg/ml) and four parts of 0.5% carboxymethoxy cellulose solution. The survival time of tumor-bearing mice were observed for 40 days and the mean survival time (days) of drug-treated mice was compared with that of the control. Six mice were used for each determination.

## Results

### Taxonomy of the Producing Organism

On CZAPEK's agar colonies grew to 30~35 mm in diameter after 10 days incubation at 27°C. The colonies were floccose and white to yellowish in color; reverse was white; no soluble pigment was observed. Sporulation on CZAPEK's agar was scant; good on malt agar and PDA, and grew more rapidly than on CZAPEK's agar (45~50 mm and 50~65 mm in diameter on malt agar and PDA, respectively after 10 days incubation at 27°C). The colonies on malt extract agar and PDA were also floccose and white to yellow to yellowish green; reverse was yellow to yellowish brown. On Sabouraud agar, the colonies were predominantly light green; reverse was green. Conidiophores were unbranched and perpendicular to funicular hyphae. In the marginal area, they were formed directly from the surface hyphae, bearing biverticillate and symmetric penicilli with colorless conidial heads. Conidiophore length and diameter, below the penicillus, ranged 40 to 60 $\mu$m$\times$2.5 to 3.0 $\mu$m. The conidia, as well as conidiophores, were smooth. Conidia formed chains at the top of phialides. Conidia were ovoid to subglobose in shape and 2.0$\times$2.5 $\mu$m in size. Sclerotia or cleistothecia were absent.

The above characteristics, serve to identify the producing organism as the genus *Penicillium*[2,3], although the species of this strain should be resolved in further studies.

A culture of this strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan with an accession number of FERM P-9437.

### Production and Isolation of Methylenolactocin

Fermentation was performed as described in Materials and Methods. As most of the antibiotic activity was found in the broth filtrate, the filtrate (20 liters) was adjusted to pH 3.0 with HCl and the

active principles were extracted with ethyl acetate. The combined ethyl acetate extract was concentrated to about 700 ml *in vacuo* (at 40°C) and extracted twice with the same volume of 5% $NaHCO_3$ (pH 9.0). The buffer layer, readjusted to pH 3.0 with HCl, was extracted twice with the same volume of ethyl acetate. The ethyl acetate extracts were dried with anhydrous sodium sulfate and concentrated *in vacuo*. The residue (*ca.* 2.5 g) was subjected to silica gel column chromatography (Fusica gel BW-820 MN). The column (29×38 mm) was developed with a solvent system of benzene - ethyl acetate (95 : 5). The elution was monitored by TLC and detected under UV lamp. Active fractions were combined, concentrated *in vacuo* and rechromatographed on silica gel column with a solvent system of benzene - ethyl acetate (95 : 5). After recrystallization from *n*-hexane - ethyl acetate, the pure antibiotic was obtained as a colorless leaflet. Yield was about 90~100 mg from 1 liter of broth. Isolation procedure is shown in Fig. 1.

## Physico-chemical Properties of Methylenolactocin

Methylenolactocin (**1**) was obtained by recrystallization from *n*-hexane and ethyl acetate as crystals of mp 82.5~83.5°C. The molecular formula of **1** was determined to be $C_{11}H_{16}O_4$ by mass spectrometry (Fig. 2) and elementary analysis; this was further confirmed by the high resolution mass spectrum (*m/z* 212.1048 ($M^+$), calcd for $C_{11}H_{16}O_4$: 212.1044). The IR spectrum is shown in Fig. 3. Also, the $^1H$ and $^{13}C$ NMR spectra are shown in Figs. 4 and 5, respectively. The Rf values on TLC using several developing solvents are shown in Table 1. Physico-chemical properties of **1** are summarized in Table 2.

Fig. 1. Isolation procedure of methylenolactocin.

Broth filtrate (20 liters)
- adjusted to pH 3.0 with HCl
- extracted with EtOAc

Organic phase
- evaporated *in vacuo*
- extracted with 5% $NaHCO_3$ (pH 9.0)

Water phase
- adjusted to pH 3.0 with HCl
- extracted with EtOAc

Organic phase
- evaporated *in vacuo*

Crude paste (*ca.* 2.5 g)
- silica gel column chromatography
- eluted with benzene - EtOAc (95 : 5)

Active fractions
- concentration *in vacuo*
- rechromatography with benzene - EtOAc (95 : 5)

Methylenolactocin (1.8 g)

## Structure of Methylenolactocin

Methylenolactocin (**1**) is an acidic substance and has a carboxylic acid judging from IR spectrum (3360 (br), 3050 (br), and 1710 $cm^{-1}$), $^1H$ NMR spectrum ($\delta$ 10.77 (1H, br s)) and $^{13}C$

Fig. 2. Mass spectrum of **1**.

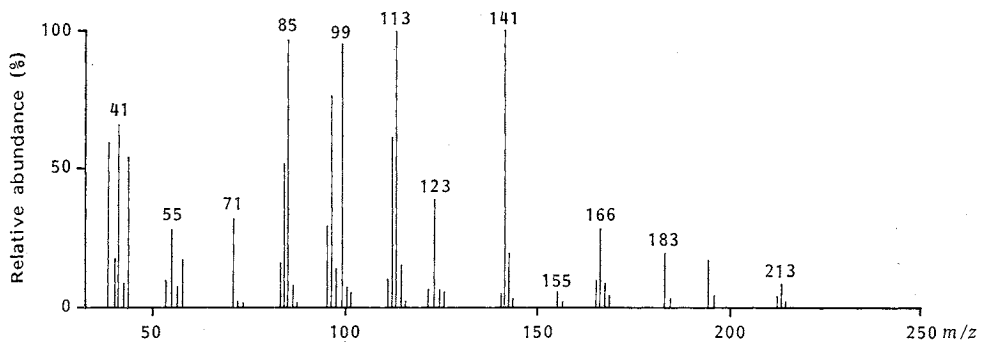


Fig. 3. IR spectrum of **1** (KBr).

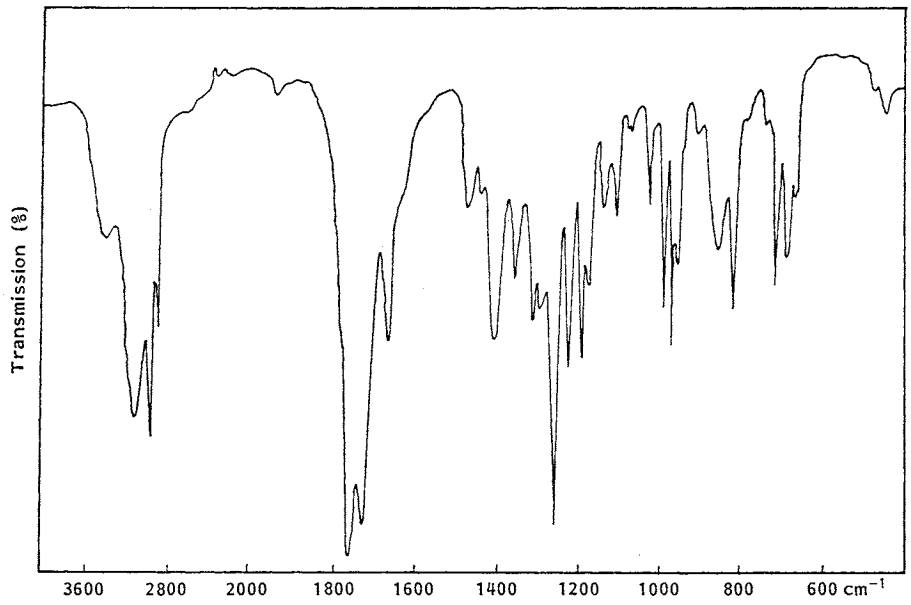
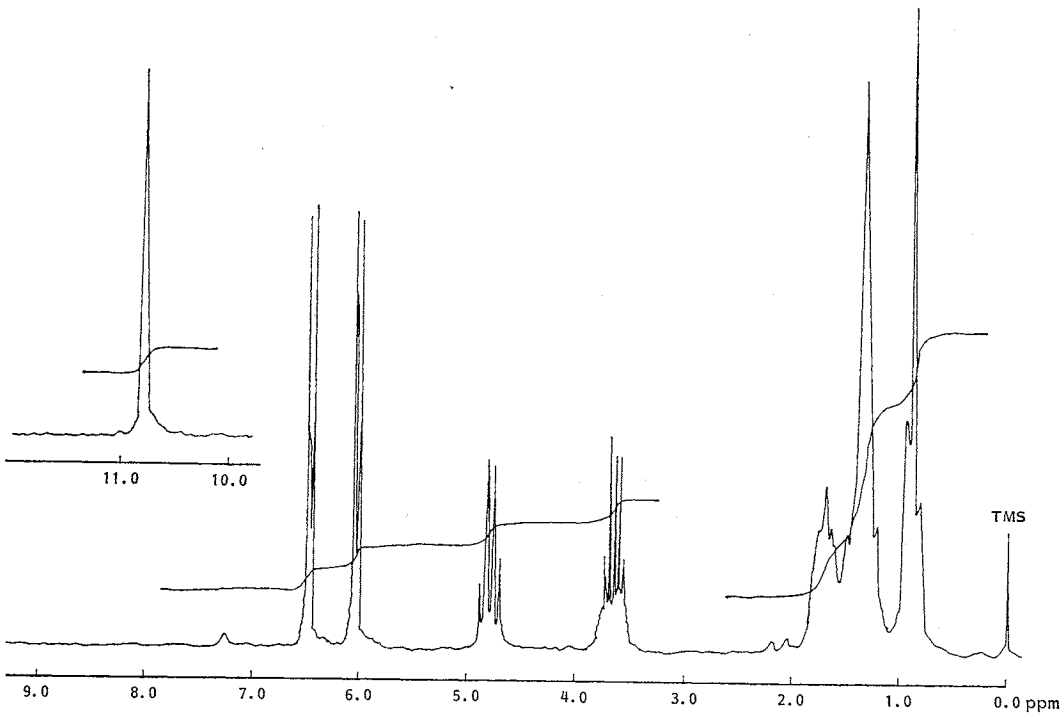
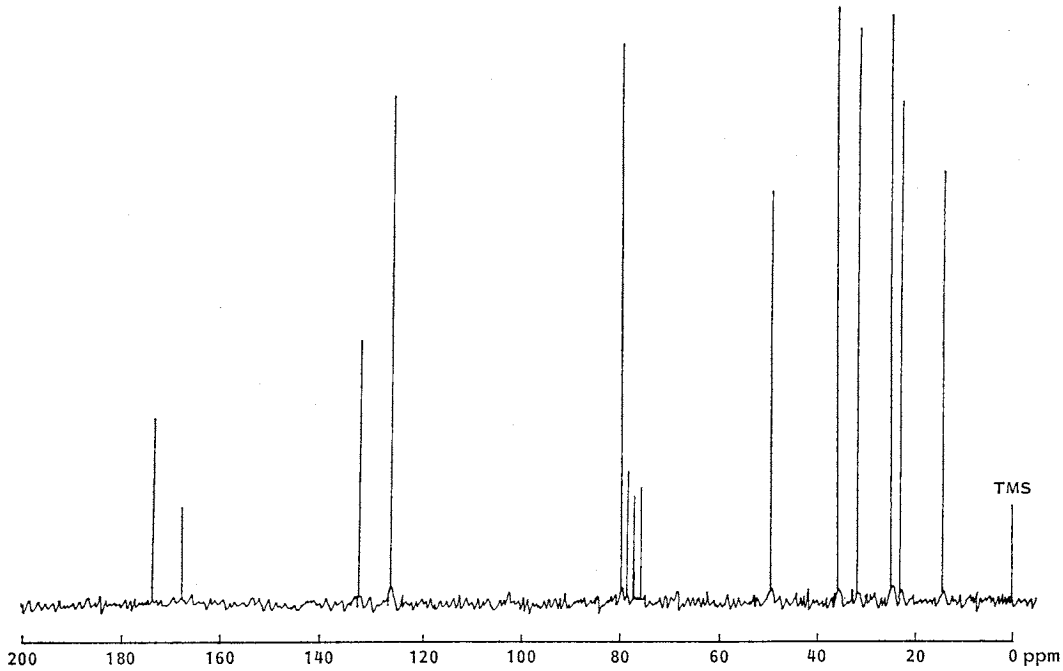



Fig. 4. $^1$H NMR spectrum of **1** (100 MHz, $CDCl_3$).




NMR spectrum ($\delta$ 174.3, s). Also, the dehydration ion peak of **1** (*m/z* 194, $M-H_2O$) was observed in the mass spectrum. The absorption band at 1740 $cm^{-1}$ in the IR spectrum indicated the presence of a lactone structure and four oxygens in **1** were assumed to form two carboxyl groups. Two of four oxygens in **1** were thought to form a lactone ring judging from $^1$H NMR spectrum and degree

Fig. 5. $^{13}C$ NMR spectrum of **1** (25 MHz, $CDCl_3$).




of unsaturation derived from the molecular formula of **1**. The $^{13}C$ NMR spectrum of **1** showed the presence of 11 carbons containing one methyl carbon, four methylene carbons, two methine carbons, two olefinic carbons, one carbonyl carbon (CO) and one carboxy (CO) carbon (Table 3).

Table 1. TLC behavior of methylenolactocin.

| Solvent system[a] | Rf values |
|---|---|
| $CHCl_3$ - EtOAc (9 : 1) | 0.31 |
| Benzene - MeOH (8 : 2) | 0.52 |
| $CHCl_3$ - acetone - EtOAc (35 : 60 : 5) | 0.60 |

TLC; Merck Silica gel plates 60 $GF_{254}$ (0.25 mm) were used and the spots were detected under UV lamp or by spraying 1% $KMnO_4$.

[a] All solvent systems contained 2% of acetic acid.

In the $^1H$ NMR spectrum, two doublets at $\delta$ 6.04 and 6.47 were attributable to exomethylene protons and this assignment was supported by the $^{13}C$ NMR spectrum ($\delta$ 126.1 (t) and 132.5 (s)) and IR spectrum (1660 $cm^{-1}$). When the proton at $\delta$ 3.65 was irradiated, two doublets at $\delta$ 6.04 and 6.47 were changed to singlets and the proton at $\delta$ 4.83 was changed from a quartet to a triplet. Considering the chemical shifts of these three protons, the protons at $\delta$ 6.04 and 6.47 were assigned to exocyclic methylene protons adjacent to an ester carbonyl group and the proton at $\delta$ 3.65 was a methine proton next to exocyclic double bond. After irradiation of the proton at $\delta$ 4.83, the signal of proton at $\delta$ 1.72 was changed from a multiplet to a triplet and the proton at $\delta$ 3.65 from a double triplet (ddd like dt) to a triplet (dd like t). Thus, the proton at $\delta$ 4.83 was assigned to a methine proton adjacent to an oxygen function considering the chemical shift and coupling pattern. From the spectral data described above, it was concluded that the partial structure of **1** is as shown in Fig. 6.

The $^{13}C$ NMR spectrum showed five carbons of the side chain moiety containing one methyl carbon at $\delta$ 13.9 and four methylene carbons at $\delta$ 22.4, 24.4, 31.3, 35.7. In the $^1H$ NMR spectrum, eleven protons appeared at $\delta$ 0.90 (3H, t), 1.36 (6H, m) and 1.72 (2H, m). Also base ion peak (*m/z*

Table 2. Physico-chemical properties of methylenolactocin.

| | | |
|---|---|---|
| Nature | Acidic, colorless leaflet | |
| MP (°C) | 82.5~83.5 | |
| Optical rotation | $[\alpha]_D^{26}$ −2.37° (*c* 3.0, MeOH) | |
| Formula | $C_{11}H_{16}O_4$ | |
| *Anal* | Found: | C 62.14, H 7.55 |
| | Calcd for $C_{11}H_{16}O_4$: | C 62.25, H 7.60 |
| EI-MS ($M^+$) | Found: | *m/z* 212.1048 |
| | Calcd for $C_{11}H_{16}O_4$: | *m/z* 212.1044 |
| UV $\lambda_{max}^{MeOH}$ nm | 210, 261 (sh), 268 (sh) | |
| IR $\nu_{max}^{KBr}$ $cm^{-1}$ | 3360, 3050, 2930, 1740, 1710, 1660, 1460, 1400, 1350, 1250, 960 | |
| Solubility Soluble: | MeOH, acetone, EtOAc, ethyl ether, $CHCl_3$, benzene | |
| Insoluble: | Water, *n*-hexane | |
| Color reactions Positive: | $KMnO_4$, $I_2$ vapor | |
| Negative: | Dragendorff, ferric chloride, ninhydrin, 2,6-dichloroindophenol, 2,4-DNPH | |

EI-MS: Electron impact mass spectrum.

Table 3. Assignment of $^{13}C$ NMR spectrum of methylenolactocin (25 MHz in $CDCl_3$).

| Peak No. | ppm | Multiplicity | Assignment |
|---|---|---|---|
| 1 | 13.9 | q | C-9 |
| 2 | 22.4 | t | C-6 or C-7 or C-8 |
| 3 | 24.4 | t | C-6 or C-7 or C-8 |
| 4 | 31.3 | t | C-6 or C-7 or C-8 |
| 5 | 35.7 | t | C-5 |
| 6 | 49.6 | d | C-3 |
| 7 | 79.2 | d | C-4 |
| 8 | 126.1 | t | C-10 |
| 9 | 132.5 | s | C-2 |
| 10 | 168.6 | s | C-1 |
| 11 | 174.3 | s | C-11 |

Table 4. Assignment of $^1H$ NMR spectrum of methylenolactocin (100 MHz in $CDCl_3$).

| ppm | Assignment |
|---|---|
| 0.90 | 9-H (3H, t, *J*=5.9 Hz) |
| 1.36 | 6-H, 7-H, 8-H (6H, m) |
| 1.72 | 5-H (2H, m) |
| 3.65 | 3-H (1H, dt, *J*=5.6, 2.9 Hz) |
| 4.83 | 4-H (1H, q, *J*=5.6, 5.4 Hz) |
| 6.04 | 10-H (1H, d, *J*=2.9 Hz) |
| 6.47 | 10-H (1H, d, *J*=2.9 Hz) |
| 10.77 | 11-H (1H, s) |

Fig. 6. Partial structure of methylenolactocin.

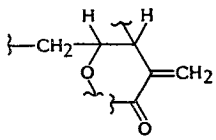
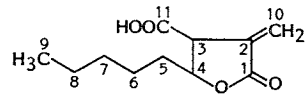


Fig. 7. Structure of methylenolactocin (**1**).



141, M−($CH_2CH_2CH_2CH_2CH_3$)) appeared in the mass spectrum. One proton remained from 16 protons, appeared at $\delta$ 10.77 (1H, s) in the $^1H$ NMR spectrum assigned to hydroxy proton. Assignment of $^1H$ NMR spectrum of **1** is summarized in Table 4.

In order to determine whether the two methine protons in the lactone ring are in the *trans* or *cis*-position, the $^1H$ NMR spectrum of **1** was compared with that of protolichesterinic acid[4], nephrosterinic acid[5] from the lichens and canadensolide[6] from the *Penicillium canadense*. Judging from coupling constants and chemical shifts, it was assumed that **1** had the *trans*-position.

However, the absolute structure of **1** is not yet completed and in progress. Thus, the structure of **1** was determined to be 3-carboxy-2-methylene-4-nonanolide as shown in Fig. 7.

Table 5. Antimicrobial spectrum of methylenolactocin.

| Organism | MIC ($\mu$g/ml) | Organism | MIC ($\mu$g/ml) |
|---|---|---|---|
| *Staphylococcus aureus* IFO 3060 | 6 | *Alcaligenes faecalis* IFO 13111 | >200 |
| *Bacillus subtilis* IFO 12210 | 100 | *Klebsiella pneumoniae* IFO 3317 | >200 |
| *B. brevis* IFO 3331 | 25 | *Penicillium chrysogenum* IFO 4897 | 100 |
| *B. cereus* IFO 3514 | 50 | *P. notatum* | 100 |
| *Micrococcus roseus* IFO 3764 | 6 | *P. urticae* IFO 7011 | 100 |
| *M. luteus* IFO 3333 | 6 | *P. experimentum* | 100 |
| *M. luteus* IFO 12708 | 100 | *Aspergillus niger* IFO 4416 | >200 |
| *Corynebacterium xerosis* IFO 12684 | 6 | *A. oryzae* | >200 |
| *Arthrobacter simplex* IFO 12069 | 100 | *Fusarium oxysporum* IFO 5880 | >200 |
| *Escherichia coli* K-12 IFO 3301 | >200 | *Mucor javanicus* | >200 |
| *E. coli* B IFO 13168 | >200 | *Saccharomyces cerevisiae* | >200 |
| *Pseudomonas aeruginosa* IFO 3923 | >200 | *Saccharomycopsis lipolytica* IFO 0746 | >200 |
| *P. putida* IFO 3738 | >200 | *Candida albicans* | >200 |
| *Proteus vulgaris* IFO 3851 | 100 | *C. tropicalis* IFO 0589 | >200 |
| *Serratia marcescens* IFO 12648 | >200 | *C. utilis* IFO 0396 | >200 |

### Biological Properties of Methylenolactocin

Results of the examination of the antimicrobial activity of methylenolactocin are shown in Table 5. It showed selective antibacterial activity, especially against Gram-positive bacteria including *Bacillus*, *Micrococcus*, *Staphylococcus* and *Corynebacterium*. Gram-negative bacteria were insensitive to **1**. Fungi and yeasts were not affected by concentration up to 200 $\mu$g/ml except some strains of *Penicillium*. The antitumor activity of methylenolactocin on Ehrlich carcinoma is shown in Table 6. The intraperitoneal injection of this antibiotic at a dose of 0.2 mg per mouse caused a prolongation of the life span of the treated mice bearing tumor cells at a level of 166.2 T/C (%) (see footnote to Table 6).

Table 6. Antitumor activity of methylenolactocin to mice inoculated with Ehrlich carcinoma.

| Dose (mg/mouse) | MSD[a] (days) | Evaluation[b] T/C (%) |
|---|---|---|
| 0.05 | 13.8±1.4 | 106.2 |
| 0.1 | 15.2±1.3 | 116.9 |
| 0.2 | 21.6±2.0 | 166.2 |
| Mitomycin 0.02 | 32.2±2.6 | 247.7 |
| Control | 13.0±1.0 | — |

$2\times10^6$ of Ehrlich carcinoma cells were inoculated intraperitoneally into each ICR mouse (female, 5 weeks old) and ethanol solution of methylenolactocin was administered intraperitoneally once every day for 10 days, starting on the day of the tumor cell inoculation.

[a] MSD: Mean survival days.

[b] T/C (%)={MSD (treated)/MSD (control)}×100.

## Discussion

Methylenolactocin is active against Gram-positive bacteria and showed antitumor activity against Ehrlich carcinoma. Methylenolactocin was established to be a new acidic antibiotic possessing an $\alpha$-exomethylene lactone moiety; however, the absolute structure has not been clarified.

Several $\alpha$-exomethylene lactone-class of natural products have been reported[7~11].

To the best of our knowledge, however, this is the first report of this class of antibiotic as a microbial product. The antimicrobial activity of methylenolactocin disappeared when it was reduced by catalytic hydrogenation. It was known that the olefinic double bond seems to be the active center of $\alpha$-exomethylene lactone-class antibiotics reacting with the sulfhydryl group of the receptor protein[12~15]. However, the mechanism of action of **1** remains to be clarified.

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