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# Decatromicins A and B, New Antibiotics Produced by *Actinomadura* sp. MK73-NF4

## I. Taxonomy, Isolation, Physico-chemical Properties and Biological Activities

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New antibiotics designated decatromicins A and B were isolated from the culture broth of *Actinomadura* sp. MK73-NF4. They were purified by butyl acetate extraction, silica gel column chromatography, silica gel TLC and Sephadex LH-20 column chromatography. Decatromicins A and B inhibited growth of Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA).

During our screening for new antibiotics, we have isolated new antibiotics, decatromicins which were produced by *Actinomadura* sp. MK73-NF4 (Fig. 1). Decatromicins A and B had a macrocyclic lactone structure containing a tetronic acid, similar to kijanimicin[1], saccharocarcins[2,3] and pyrrolosporin A[4,5]. Decatromicins showed antimicrobial activities against Gram-positive bacteria and were not active against Gram-negative bacteria.

In this paper, the taxonomy of the producing strain, isolation, physico-chemical properties and biological activities of decatromicins A and B are reported. The structural studies of decatromicins A and B will be described in the following paper[6].

### Materials and Methods

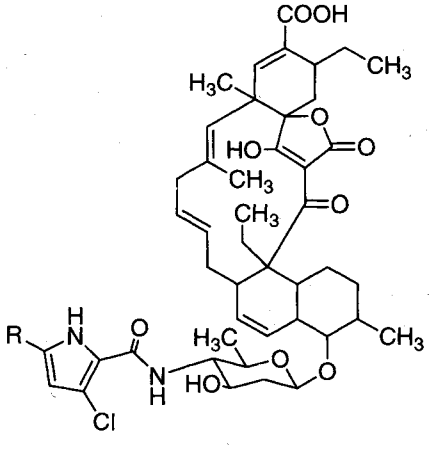
Microorganism

The decatromicins A and B producing strain, MK73-NF4 was isolated from a soil sample collected at Itano-gun, Tokushima prefecture, Japan. This strain have been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan under the accession number FERM P-16335.

Taxonomic Studies

Morphological characteristics of the spores and mycelia were observed with a scanning electron microscope

Fig. 1. The structure of decatromicins A and B.




Decatromicin A : R=H
Decatromicin B : R=Cl

(Hitachi S-570). Cultural and physiological characteristics were determined by the methods of SHIRLING and GOTTLIEB[7], and WAKSMAN[8]. The substrate and aerial mycelium color including soluble pigments were assigned by the Color Harmony Manual, 1958 (Container Corporation of America, Chicago). Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[9]. 2,6-Diaminopimelic acid in the cell wall was analyzed by the method of BECKER *et al.*[10] and STANECK and ROBERTS[11]. Whole-cell sugars were determined by the method of LECHEVALIER and LECHEVALIER[12], and MIKAMI and ISHIDA[13]. Phospholipids were analyzed by the procedure of MINNIKIN *et al.*[14]. Menaquinones were analyzed by HPLC and mass spectrometry as described by TAMAOKA *et al.* [15].

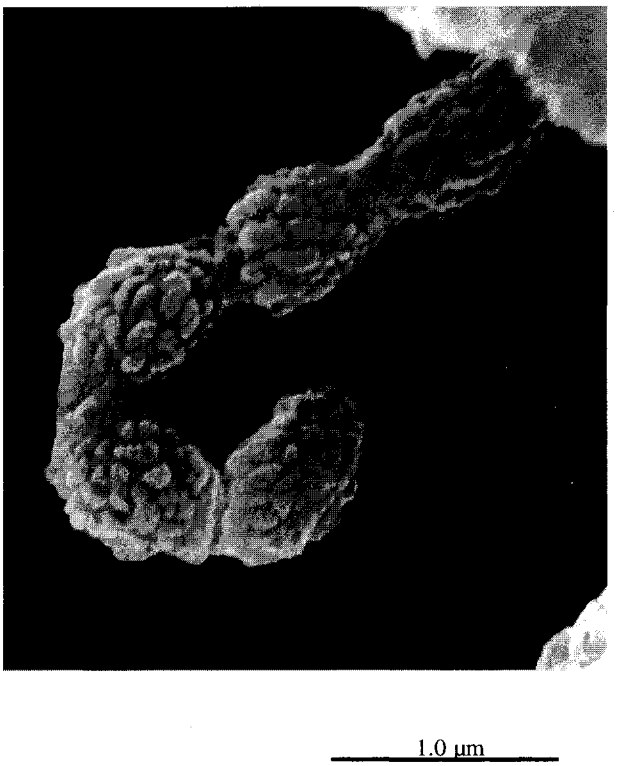
Production of Decatromicins A and B

A slant culture of strain MK73-NF4 was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of preculture medium consisting of glycerol 2.0%, dextrin 2.0%, Bacto-soytone (Difco) 1.0%, yeast extract 0.3%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% and a drop of silicon oil (Shin-Estu Chemical Industry, KM-70) (pH 7.4 before sterilization). The flask was shaken on a rotary shaker (180 rpm) at 30°C for 96 hours. This preculture (3 ml) was transferred into 500-ml Erlenmeyer flasks each containing 110 ml of the production medium consisting of meat extract 0.3%, yeast extract 0.5%, tryptose 0.5%, glucose 1.0% and Bacto-ager (Difco) 0.15% (pH 6.8~7.0 before sterilization). Production of decatromicins was carried out at 27°C for 168 hours on a rotary shaker. In the case of production of decatromicins A and B using jar-fermentor, this preculture (550 ml) was transferred into 30-liters jar-fermentor containing 15 liters of the same production medium with a small amount of silicon oil (Toho kagaku, Pronal 502). The fermentation was incubated for 168 hours under the following condition: temperature; 27°C, agitation; 200 rpm/minute, aeration; 15 liters/minute. The production of decatromicins in the culture was monitored by the determination of the antimicrobial activity.

HPLC Analysis

The amount of decatromicins A and B were measured by reverse phase HPLC under the following condition: column; PEGASIL ODS (Senshu Scientific Co., Ltd., 4.6×150 mm), mobile phase; $CH_3CN$ - $H_2O$ (3 : 1) containing 0.1% $CH_3COOH$, flow rate; 1.0 ml/minute, detection; UV at 270 nm. Under these conditions, decatromicins A and B were eluted at 4.66 and 8.75 minutes, respectively.

Fig. 2. Scanning electron micrograph of strain MK73-NF4 on sucrose-nitrate agar after incubation at 30°C for 21 days.




Biological Activities

Antimicrobial activities of decatromicins A and B were assayed by the cup or paper-disk diffusion methods against *Bacillus stearothermophilus*. The MICs of decatromicins A and B against bacteria and yeast were determined by the agar dilution method in Mueller-Hinton agar (Difco).

## Results and Discussion

Taxonomy of the Decatromicins A and B Producing Strain

Strain MK73-NF4 formed well-branched vegetative mycelia. The strain formed aerial hyphae with short spore chains in the shape of loop and hook. The mature spore chain consisted of 3 to 10 spores. The spores were oval (0.6~0.8×0.8~1.0 μm) and not motile. Their spore surfaces were warty as shown in Fig. 2. No synnemata, sclerotia or sporangia were observed. The cultural characteristics of strain MK73-NF4 on various agar media are shown in Table 1. The color of growth was colorless on sucrose - nitrate agar and inorganic salts - starch agar (ISP

Table 1. Cultural characteristics of strain MK73-NF4.

| Medium | Growth | Aerial mycelium | Soluble pigment |
|---|---|---|---|
| Sucrose-nitrate agar | Colorless | None | None |
| Yeast extract-malt extract agar (ISP No. 2) | Pale pink [5gc, Peach Tan~5ic, Lt Persimmon] | Scant, white | None |
| Oatmeal agar (ISP No. 3) | Pale pink [5ea, Peach Pink] | Scant, white | None |
| Inorganic salts-starch agar (ISP No. 4) | Colorless | Thin, white | None |
| Glycerol-asparagine agar (ISP No. 5) | Pale yellow [2ca, Lt Ivory] | None | None |
| Tyrosine agar (ISP No. 7) | Yellowish gray [3ca, Pearl Pink] ~Pale pink [5ca, Flesh Pink] | None | None |

Observation after incubation at 27℃ for 21 days.

Table 2. Physiological characteristics of strain MK73-NF4.

| | | Utilization of | |
|---|---|---|---|
| Temperature range for growth (℃) | 20 ~ 37 | L-Arabinose | - |
| Optimum temperature (℃) | 30 | D-Fructose | - |
| Formation of melanoid pigment | - | D-Glucose | + |
| Hydrolysis of starch | - | Inositol | - |
| Reduction of nitrate | + | D-Mannitol | - |
| | | Raffinose | - |
| | | Rhamnose | + |
| | | Sucrose | - |
| | | D-Xylose | - |

+ : positive; - : negative

No. 4), pale pink on yeast extract-malt extract agar (ISP No. 2) and oatmeal agar (ISP No. 3), pale yellow on glycerol-asparagine agar (ISP No. 5), and yellowish gray on tyrosine agar (ISP No. 7). The color of aerial mycelia was white on ISP media Nos. 2, 3 and 4. Soluble pigments were not produced. The physiological properties of strain MK73-NF4 are shown in Table 2.

An analysis of cell wall hydrolysate of the strain revealed the presence of *meso*-diaminopimelic acid. The whole-cell hydrolysate of the strain showed the presence of madurose as a diagnostic sugar. These data indicated that strain MK73-NF4 had a type III cell wall and a type B whole-cell sugar pattern. The phospholipid type was PI, which contained phosphatidylglycerol and phosphatidylinositol, but none of phosphatidylethanolamine, phosphatidylcholine or unknown glucosamine-containing phospholipids. The major menaquinone was MK-9 ($H_6$) and the minor one was MK-9 ($H_8$).

On the basis of these characteristics, strain MK73-NF4 was found to belong to the genus *Actinomadura*[16,17].

## Isolation

The culture broth (5 liters) was centrifuged to separate the supernatant and the mycelium cake. The supernatant (4.2 liters) was extracted with buthyl acetate (4.2 liters).

Table 3. Physico-chemical properties of decatromicins A and B.

| | Decatromicin A | Decatromicin B |
|---|---|---|
| Appearance | White powder | White powder |
| MP | 223~ 225 ℃ (dec.) | 202~ 206 ℃(dec.) |
| $[\alpha]_D^{26}$ | +2.0 ° ( *C* 1.30, MeOH) | -9.0 ° ( *C* 1.30, MeOH) |
| Molecular formula | $C_{45}H_{57}ClN_2O_{10}$ | $C_{45}H_{56}Cl_2N_2O_{10}$ |
| FAB-MS (*m/z* ) | 821 $(M+H)^+$ | 855 $(M+H)^+$ |
| | 819 $(M-H)^-$ | 853 $(M-H)^-$ |
| HRFAB-MS (*m/z* ) | | |
| Calcd: | 821.3780 (as $C_{45}H_{58}ClN_2O_{10}$) | 855.3390 (as $C_{45}H_{57}Cl_2N_2O_{10}$) |
| Found: | 821.3801 $(M+H)^+$ | 855.3364 $(M+H)^+$ |
| UV $\lambda$ max nm, (log $\varepsilon$ ) in | | |
| MeOH | 271 (4.47) | 269 (4.46) |
| MeOH-NaOH | 241 (4.22), 285 (4.40) | 240 (4.25), 285 (4.50) |
| MeOH-HCl | 270 (4.44) | 268 (4.43) |
| IR $\nu$ maxKBr cm-1 | 3440, 2960, 2930, 1765, 1695, 1625, 1560, 1525, 1450, 1330, 1220, 1170, 1130, 1070, 1025, 970 | 3400, 2960, 1760, 1690, 1640, 1550, 1530, 1440, 1330, 1240, 1170, 1140, 1080, 1025, 975 |
| TLC (Rf value)[a] | 0.23 | 0.29 |

[a] Silica gel TLC (Merck Art. 105715) : $CHCl_3$-MeOH-$CH_3COOH$ (20 : 1 : 0.03)

The buthyl acetate extract was evaporated to dryness to give a brown oil (422.6 mg). This oily substance was chromatographed on a silica gel column (Wakogel C-200) and eluted stepwise with mixtures of $CHCl_3$ - MeOH (100 : 0, 50 : 1, and 10 : 1). The active fractions were eluted with the mixture of $CHCl_3$ - MeOH (50 : 1). The fractions were collected and concentrated under reduced pressure to give a pale yellow solid (280.8 mg). The crude antibiotics were isolated by silica gel TLC (Merck Silica gel $60F_{254}$ Art. 105715, $CHCl_3$ - MeOH - $CH_3COOH$, 20 : 1 : 0.03) providing crude decatromicin A (39.8 mg) and B (213.4 mg). The crude decatromicins were separately purified by a Sephadex LH-20 column chromatography developed with the mixture of $CHCl_3$ - MeOH (1 : 1) to give pure decatromicin A (31.7 mg) and decatromicin B (154.7 mg).

## Physico-chemical Properties

Physico-chemical properties of decatromicins A and B are shown in Table 3. The compounds were soluble in MeOH and DMSO, slightly soluble in $CHCl_3$ and EtOAc, but insoluble in hexane and water. The molecular formula for decatromicins A and B were determined to be $C_{45}H_{57}ClN_2O_{10}$ and $C_{45}H_{56}Cl_2N_2O_{10}$ by HRFAB-MS, respectively. The UV spectra showed absorption maxima at 271 and 269 nm in MeOH, respectively. The UV spectra of decatromicins A and B were similar to that of pyrrolosporin A. Decatromicins gave a positive color reaction with molybdophosphoric acid-sulfuric acid, 2,4-dinitrophenylhydrazine, Rydon-Smith and anisaldehyde-sulfuric acid reagents, but a negative one with ninhydrin reagent.

## Biological Activities

The antimicrobial activities of decatromicins A and B are shown in Table 4. Decatromicins A and B inhibited the growth of Gram-positive bacteria including multi-drug resistant strains such as *Staphylococcus aureus* MS9610 and methicillin-resistant *S. aureus* (MRSA). The antimicrobial activity of decatromicin B was stronger than that of decatromicin A. This obsevation suggested that antibacterial activity against Gram-positive bacteria of decatromicins might increase with increase in the numbers of chlorine atom attached to the pyrrole ring. However these antibiotics did not inhibit growth of Gram-negative bacteria and yeast at 100 $\mu$g/ml.

The acute toxicity ($LD_{50}$) of decatromicins A and B in mice (i.p.) were estimated to be more than 100 mg/kg.

Table 4. The antimicrobial activities of decatromicins A and B.

| Test organism | MIC(μg/ml) | |
|---|---|---|
| | Decatromicin A | Decatromicin B |
| *Staphylococcus aureus* FDA209P | 1.56 | 0.39 |
| *S. aureus* Smith | 1.56 | 0.78 |
| *S. aureus* MS9610 | 3.13 | 0.39 |
| *S. aureus* MS16526 (MRSA) | 1.56 | 0.39 |
| *S. aureus* TY-04282 (MRSA) | 1.56 | 0.78 |
| *Micrococcus luteus* IFO3333 | 3.13 | 0.78 |
| *Bacillus subtilis* PCI219 | 3.13 | 0.78 |
| *Corynebacterium bovis* 1810 | 12.5 | 6.25 |
| *E.coli* K-12 | >100 | >100 |
| *Shigella dysenteriae* JS11910 | >100 | >100 |
| *Salmonella typhi* T-63 | >100 | >100 |
| *Proteus vulgaris* OX19 | >100 | >100 |
| *Providencia rettgeri* GN311 | >100 | >100 |
| *Serratia marcescens* | >100 | >100 |
| *Pseudomonas aeruginosa* A3 | 100 | 100 |
| *Klebsiella pneumoniae* PCI602 | >100 | >100 |
| *Mycobacterium smegmatis* ATCC607 [a] | >100 | >100 |
| *Candida albicans* 3147 | >100 | >100 |

Mueller Hinton agar (Difco) 37℃ 18 hours.

[a] 37℃ 42 hours.

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