# Madindoline, a Novel Inhibitor of IL-6 Activity from *Streptomyces* sp. K93-0711

## I. Taxonomy, Fermentation, Isolation and Biological Activities

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(Received for publication June 3, 1996)

Selective growth inhibition against IL-6-dependent cells was detected in fermentation extracts of a microbial strain, K93-0711, which was characterized as *Streptomyces* species. Active metabolite, termed madindoline A and B, were isolated, and the structure was determined to be 3a-hydroxyindoline with diketocyclopentene at the *N* position. Madindoline A and B displayed dose-dependent inhibition of MH60 cells, an IL-6-dependent cell line, in presence of 0.1 U/ml IL-6. The $IC_{50}$ for madindoline A and B against this cell line was 8 $\mu$M and 30 $\mu$M, respectively. These compounds did not inhibit the growth of cell lines which are not IL-6 dependent and the growth inhibition of the MH60 cell line was reversed by addition of excess, 0.4 U/ml, of IL-6 to the culture media. These compounds did not show any antimicrobial activity at a concentration of 1,000 $\mu$g/ml.

Cytokines play important roles in cancer[1], inflammation[2] and immune response. Therefore, cytokine inhibitors may be, potentially effective against cancer and chronic or refractory inflammatory diseases. IL-6 is a multifunctional cytokine involved in the regulation of immune reaction[3], hematopoiesis[4] and acute-phase response[5], and the growth of certain types of tumor cells[6]. Thus, it is possible that a substance which inhibit growth of IL-6-dependent tumor cells will possess selective activity against tumors resulting from these cells.

In the course of a screening program aimed at IL-6 inhibitors obtained from microorganisms, two compounds were discovered in the fermentation broth of *Streptomyces* sp. K93-0711, and were designated as madindoline A and B. The present paper describes taxonomic studies of the producing strain, and the production, isolation and biological activity of the new compound, madindoline.

## Materials and Methods

### General Experimental Procedures

The microbial strain K93-0711 isolated from a soil sample was used for production of madindoline A. DC-Alufolien Kieselgel 60 $F_{254}$ (Merck) was used for TLC analysis. HPLC was carried out using the HITACHI D-2000 system and an ODS packed column (Senshu Pak Pegasil ODS, 5 $\mu$m, i.d. 20 × 250 mm).

### Taxonomic Studies

The morphological properties were observed with a scanning electron microscope (model S-430, Hitachi Co., Ltd.). The isomer of diaminopimelic acid (DAP) was determined by the method of TAKAHASHI *et al.*[7]. In order to investigate the cultural characteristics and physiological properties, the International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTEIEB[8] and media recommended by WAKSMAN[9] were used. Cultures were observed after incubation at 27°C for 2 weeks. The color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago)[10] was used for color names and hue numbers. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium[11] containing a 1% carbon source at 27°C.

### Antimicrobial Activity

Antimicrobial activity was tested using paper disks (i.d. 6 mm, ADVANTEC). Bacteria were grown on Mueller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato broth agar medium. Antimicrobial activity was observed after 24-hour incubation at 37°C for bacteria and after 48-hour incubation at 27°C for fungi and yeasts.

### Cytotoxic Activity Tests

Eight cell lines were maintained in monolayers or in suspension in EAGLE's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) or RPMI 1640 medium supplemented with 10% FCS. In order to determine the cytotoxicity of madindoline, cells suspended in 200 $\mu$l of the medium were plated in

a 96-well culture plate (Corning) and incubated for 24 hours at 37°C in a 5% $CO_2$-95% air atmosphere. Five $\mu$l of medium containing different concentrations of madindoline A and B was added to each well. After 72 hours incubation, the cell growth was measured colorimetrically using the tetrazolium salt (MTT) method[12].

Cytotoxicity was measured using stock cultures of MH60 cells washed twice with RPMI 1640 medium in order to remove recombinant human IL-6 (rhIL-6: Wako) completely. Washed cells ($5 \times 10^3$ cells) were suspended in 100 $\mu$l of RPMI 1640 medium containing 10% FCS and plated in a 96-well culture plate. Inoculated plates were incubated with 5 $\mu$l of test samples for 72 hours at 37°C in the presence of 0.02 U rhIL-6 (100 $\mu$l) in a 5% $CO_2$-95% air atmosphere. The cell growth was evaluated by tetrazolium method as described above.

IL-6-independent MH60 cell line was established by gradually decreasing IL-6 concentration in the medium. The growth rate of IL-6-independent cells in the medium without IL-6 was almost the same as that of IL-6-dependent cells, and the cytotoxicity on this cell line was examined by the method described above.

## Results and Discussion

### Taxonomy of Producing Strain K93-0711

Strain K93-0711 was isolated from soil collected in Madison, Wisconsin, U.S.A.

Morphological Properties:

Vegetative mycelia grew abundantly on both synthetic and complex media, and showed bacillary elements. The aerial mycelia grew abundantly on inorganic salts - starch agar and glucose - asparagine agar. The spore chains were *Rectiflexibiles* type and each had more than 20 spores per chain. The spores were cylindrical in shape, $1.4 \times 0.7$ $\mu$m in size, and had a smooth surface (Fig. 1). Whirls, sclerotic granules, sporangia and flagellated spores were not observed.

Chemical Composition:

The DAP isomer in whole-cell of strain K93-0711 was determined to be the LL-type.

Cultural Characteristics and Physiological Properties:

The cultural characteristics and the physiological properties are shown in Tables 1 and 2. The vegetative mycelia showed a brown color on various media. The

Fig. 1. Scanning electron micrograph of spore chain of strain K93-0711 grown on inorganic salts - starch agar for 14 days.

Bar represents 1 $\mu$m.

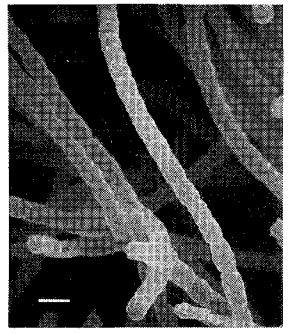


Table 1. Cultural characteristics of strain K93-0711.

| Medium | Growth | Reverse color | Aerial mass color | Soluble pigment |
|---|---|---|---|---|
| Yeast extract - malt extract agar | Good, lt. mustard tan (2ie) | Biscuit (2ec) | Abundunt, covert gray (2fe) | None |
| Oatmeal agar* | Good, bamboo (2gc) | Bamboo (2gc) | Moderate, gray (2fe) | None |
| Inorganic salts - starch agar* | Moderate, bamboo (2gc) | Lt. mustard tan (2ie) | Moderate, covert gray (2fe) | None |
| Glycerol - asparagine agar* | Good, natural (2dc) | Natural (2dc) | Abundunt, silver gray (3fe) | None |
| Glucose - asparagine agar | Moderate, pearl pink (3ca) | Pearl pink (3ca) | Poor, pearl (2ba) | None |
| Peptone - yeast extract - iron agar** | Moderate, lt. mustard tan (2ie) | Lt. mustard tan (2ie) | None | Lt. brown (3lg) |
| Tyrosine agar* | Moderate, lt. brown (3lg) | Lt. brown (3lg) | Moderate, natural (2dc) | Lt. brown (3lg) |
| Sucrose - nitrate agar** | Moderate, ivory tint (2cb) | Ivory tint (2cb) | Poor, pearl (2ba) | None |
| Glucose - nitrate agar** | Moderate, biscuit (2ec) | Bamboo (2gc) | Moderate, alabaster tint (13ba) | None |
| Glycerol - calcium malate agar | Good, covert tan (2ge) | Bamboo (2gc) | Abundant, alabaster tint ~ gray (13ba ~ i) | None |
| Glucose - peptone agar | Good, mustard tan (11/21c) | Golden brown (3pg) | Abundant, alabaster tint (13ba) | None |
| Nutrient agar | Moderate, putty (11/2ec) | Ivory (2db) | Moderate, alabaster tint ~ pearl gray (13ba ~ 13dc) | None |

* Medium recommended by International Streptomyces Project.

** Medium recommended by S. A. WAKSMAN.

Table 2. Physiological properties of strain K93-0711.

| | |
|---|---|
| Melanin formation | − |
| Tyrosinase reaction | − |
| $H_2S$ production | − |
| Liquefaction of gelatin (21°C~23°C) | + |
| Peptonization of milk (37°C) | + |
| Coagulation of milk (37°C) | − |
| Cellulolytic activity | − |
| Hydrolysis of starch | + |
| Nitrate reduction | + |
| Temperature range for growth | 15~39°C |

+, Active; −, not active.

Table 3. Utilization of carbon sources by strain K93-0711.

| | |
|---|---|
| D-Glucose | ++ |
| D-Fructose | − |
| L-Rhamnose | + |
| D-Mannitol | − |
| L-Arabinose | ++ |
| *i*-Inositol | − |
| Raffinose | − |
| D-Xylose | ++ |
| Sucrose | − |
| Melibiose | − |

++, Utilized; +, weakly utilized; −, not utilized.

aerial mass color was white to gray. Soluble pigment was produced on peptone-yeast extract-iron agar and tyrosine agar. The utilization of carbon sources is shown in Table 3.

Based on the taxonomic properties described above, strain K93-0711 is considered belong to the genus *Streptomyces*[13]. The strain was deposited in the National Institute of Bioscience and Human Technology, Japan, under the name *Streptomyces* sp. K93-0711, and the accession No. is FERM P-15253.

## Fermentation

A culture of *Streptomyces* sp. K93-0711 from Seino agar slant was inoculated into test tubes (2×20 mm) containing 10 ml of a medium consisting of 0.1% glucose, 2.4% starch, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract, 0.4% $CaCO_3$ (adjusted to pH 7.0 with 6 N NaOH before sterilization). The test tube was incubated on a reciprocal shaker at 200 rpm at 27°C for 3 days. The seed culture (5×10 ml) was inoculated into eight 500-ml Erlenmeyer flasks containing 100 ml of the same medium and then fermented under the same culture conditions. Then 700 ml of the seed culture was transferred into a 100-liter jar fermenter containing 70 liters of a production medium consisting of 2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract, 0.4% $CaCO_3$, 5 mg/ml trace metals solution (each 1 g/liter: $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$ and $CaCl_3 \cdot 6H_2O$: pH 6.7) and 0.5% Allophosite (100 mesh) (adjusted to pH 7.0 with 6 N NaOH before sterilization). The fermentation was carried out at 27°C for 4 days with agitation at a rate of 250 rpm and aeration at 10 liter/minute. The production of madindoline was monitored by HPLC analysis (column: Senshu Pak Pegasil ODS (4.6×250 mm, Senshu Scientific Co., Ltd.), solvent; 50% acetonitrile-water, flow rate: 0.8 ml/minute, detection: absorbance at 210 nm) of an ethyl acetate extract of the culture filtrate. Under these conditions, madindoline A and B were eluted at 23.7 and 25.9 minutes, respectively. After 4 days of fermentation, the amount of madindoline A and B in the broth filtrate reached a maximum of 0.17 and 0.3 μg/ml, respectively.

## Isolation and Purification

The fermentation broth (70 liters) was divided into two layers with continuous centrifugation. The inactive mycelial cake was discarded, the supernatant was extracted with EtOAc (60 liters) and than the organic layer was dried over $Na_2SO_4$. The combined EtOAc layers were concentrated under reduced pressure to give a red oil (11.5 g). The oily residue was applied to silica gel column chromatography (E. Merck, Kieselgel 60, 70~230 mesh). The materials were eluted with a step wise gradient of $CHCl_3$-$CH_3OH$ (100:0, 100:1, 50:1, 20:1, 10:1, each 4 liters). Fractions exhibiting an inhibitory effect on the growth of IL-6-dependent cells (MH60) were collected. The active fraction ($CHCl_3$-$CH_3OH$, 100:1) was concentrated *in vacuo* to yield an oily material (561.4 mg).

Further purification of madindoline was carried out by HPLC using a preparative ODS column (PEGASIL ODS (20×250 mm); Senshu Science Co. LTD., Japan; solvent: 50% aq $CH_3CN$: UV 210 nm, flow rate: 8 ml/minute). Under these conditions, madindolines were eluted at 40.1 (A) and 42.9 (B) minutes and obtained as 11.8 and 20.8 mg of light yellow powder, respectively.

Structures of madindoline A and B are shown in Fig. 2. Madindoline A and B were assumed to be stereoisomers because of the same UV absorption, molecular weight and molecular formula. Further studies on determination of the structure of madindoline A and B will be reported in a separate paper in detail.

Fig. 2. Structures of madindoline A and B.

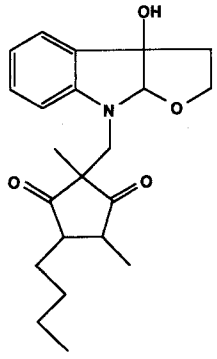
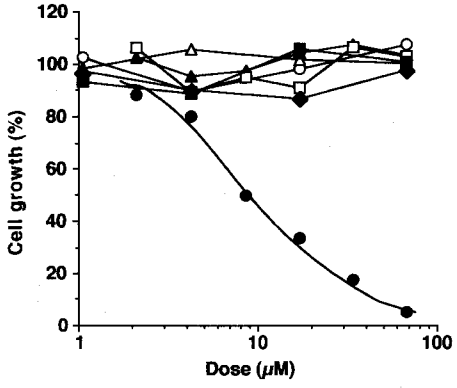



Fig. 3. Effect of madinodoline A on the growth in various cell lines.

● IL-6-MH60, ○ B16, △ P388/ADM, ▲ HUVEC, □ CPAE, ■ HL60, ◆ SC-115.




## Biological Activity

Cytocidal activity of madindoline A was examined against various mammalian cells *in vitro* (Fig. 3). The antibiotic markedly inhibited only the IL-6-induced growth of IL-6-dependent MH60 cells in a dose dependent manner, without affecting the growth of B16 melanoma, hormone-dependent Shionogi carcinoma (SC-115), adriamycin-resistant P388 leukemia (P388/ADM), calf pulmonary artery endothelial cells (CPAE), human umbilical vein endothelial cells (HUVEC) and human leukemia HL60.

Inhibitory effects of madindolines on IL-6-dependent and -independent MH60 cells were also examined. As shown in Fig. 4, these compounds dose-dependently inhibited the growth of IL-6-dependent MH60, but did not inhibit the growth of IL-6-independent MH60 cells. The $IC_{50}$ values of madindoline A and B for IL-6-dependent MH60 were 8 $\mu$M and 30 $\mu$M, respectively. These results indicate the selective inhibition of madindoline A on IL-6-induced cell growth.

Fig. 4. Effect of madindoline A (MDL-A) and B (MDL-B) on the growth of IL-6-dependent and IL-6-independent MH60 cells.

○ MDL-A-treated IL-6 dependent MH60 cell, ● MDL-A-treated IL-6 independent MH60 cell, □ MDL-B-treated IL-6 dependent MH60 cell, ■ MDL-B-treated IL-6 independent MH60 cell.

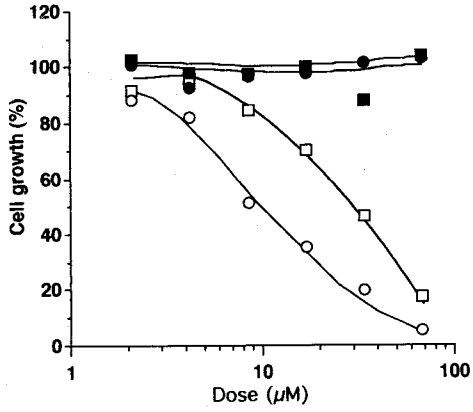
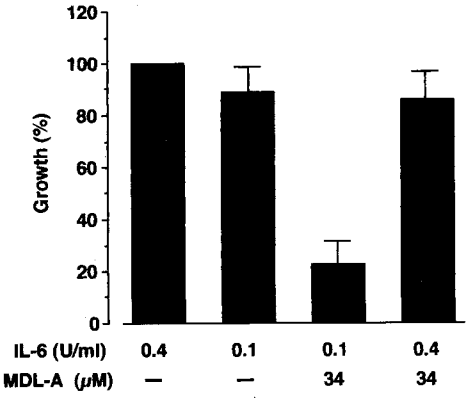



Fig. 5. Reversible effect by increased IL-6 dose on madindoline A (MDL-A)-treated IL-6 dependent MH60 cells.




The relationship between cytotoxicity of madindoline A and the concentration of IL-6 was further examined (Fig. 5). The growth inhibition of 34 $\mu$M madindoline A was avoided by the addition of excess IL-6 (0.4 U/ml) to the medium. The result suggests the competitive inhibition of IL-6 activity by madindoline A.

This antibiotic showed no antimicrobial activities at a concentration of 1,000 $\mu$g/ml against *Staphylococcus aureus* KB34 (FDA 209p), *Micrococcus luteus* KB40 (PCI 1001), *Bacillus subtilis* KB27 (PCI 219), *Mycobacterium smegmatis* KB46 (ATCC 607), *Escherichia coli* KB8 (NIHJ), KB105 (P-3), *Xanthomonas oryzae* KB88, *Candida albicans* KF1, *Saccharomyces sake* KF26, *Aspergillus niger* KF103 (ATCC 6725), *Pyricularia oryzae* KF180, *Mucor acemosus* KF223, *Clostridium perfringens* KB129, *Bacteroides fragilis* KB169, and *Acholeplasma*

*laidlawii* PG-8.

IL-6-dependent cell growth was dose-dependently and selectively inhibited by treatment with madindoline A, but, the activity of the antibiotic was completely avoided with addition of excessive IL-6 to the medium. IL-6 receptor is composed of two subunits, $\alpha$-chain (gp 80) and $\beta$-chain (gp 130), which are associated with signal transduction for proliferation or differentiation in cells[3]. Because madindoline A did not directly bind to the IL-6 molecule (data not shown), it seems likely that madindoline A interfered with the binding of IL-6 to its receptor or the transduction of receptor associated signals. To clarify the active site of madindoline A, other IL-6-induced activities and effects on other cytokines are under examination at present.

Neoplastic diseases are frequently associated with metabolic changes collectively known as cancer cachexia[14]. Tumor necrosis factor (TNF) has been suggested as a principal mediator of cancer cachexia[15]. However, recent extensive study suggests the relationship between cancer cachexia and IL-6, which is excessively produced by cancer cells and has a more significant role than TNF in mediating the many parameters of cachexia[16,17].

Acknowledgments

The authors wish to thank CHIKAKO KATO and HISASHI ORIBE for their excellent technical assistance. This work was supported in part by Grants-in-Aid from the Ministry of Health and Welfare and the Ministry of Education, Science and Culture, Japan, and by funds from the Japan Keirin Association.

## References

1) PENNICA, D.; G. E. NEDWIN & J. S. HAYFLICK: Human tumor necrosis factor; Precursor structure expression and homology to lymphotoxin. Nature 312: 724~726, 1984
2) OPPENHEIM, J. J.; C. O. C. ZACHARISE, N. MUKAIDA & K. MATSUSHIMA: Properties of the novel proinflammatory supergene "intercrine" cytokine family. Annu. Rev. Immunol. 9: 617~648, 1991
3) KISHIMOTO, T.; S. AKIRA & T. TAGA: IL-6 receptor and mechanism of signal transduction. Int. J. Immunopharmacol. 14: 431~438, 1992
4) HIRANO, T.: Interleukin 6 (IL-6) and its receptor: their role in plasma cell neoplasias. Int. J. Cell Cloning 9: 166~184, 1991
5) HOCKE, G. M.; D. BARRY & G. H. FEY: Synergistic action of interleukin-6 and glucocorticoids is mediated by the interleukin-6 response element of the rat alpha 2 macroglobulin gene. Mol. Cell Biol. 12: 2282~2294, 1992
6) ZHANG, X. G.; R. BATAILLE, M. JOURDANS, S. SAELAND, J. BANCHEREAU, P. MANNONI & B. KLEIN: Granulocyte-macrophage colony-stimulating factor synergizes with interleukin-6 in supporting the proliferation of human myeloma cells. Blood 76: 2599~2605, 1990
7) TAKAHASHI, Y.; Y. IWAI, H. NIMURA, T. KINOSHITA & S. ŌMURA: Optical resolution of 2,6-diaminopimelic acid stereoisomers by high performance liquid chromatography for the chemotaxonomy of actinomycete strains. J. Gen. Appl. Microbiol. 35: 27~32, 1989
8) SHIRLING, E. B. & D. GOTTLIEB: Method for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
9) WAKSMAN, S. A. (*Eds.*): The Actinomycetes. Classification, identification and description of genera and species. Williams and Wilkins Co. 2: 1961
10) TAYLER, H. D.; L. KNOCHE & W. C. GRANVILLE (*Eds.*): Color Harmony Manual 4th. Ed. Container Corporation of America, Chicago, 1958
11) PRIDHAM, T.G. & D. GOTTLIEB: The utilization of carbon compounds by some *Actinomycetales* as an aid for species determination. J. Bacteriol. 56: 107~114, 1948
12) ALLEY, M. C.; D. A. SCUDIERO, A. MONKS, M. L. HURSEY, M. J. CZERWINSKI, D. L. FINE, B. J. ABBOTT, J. G. MAYO, *et al.*: Feasibility of drug screening with a panel of human tumor cell lines using a microculture tetrazolium assay. Cancer Res. 48: 589~601, 1988
13) WILLAMS, S. T.; M. GOODFELLOW & G. ALDERSON: Genus *Streptomyces* Waksman and Henrici 1943. *In* BERGEY's Manual of Systemic Bacteriology. 4th Ed. S. T. WILLIAMS *et al.*, pp. 2452~2492, Williams & Wilkins Co., 1989
14) LANGSTEIN, H. N. & J. A. NORTON: Mechanisms of cancer cachexia. Hematol. Oncol. Clin. North Am.: 103~107, 1991
15) OLIFF, A.; D. DEFOS-JONES, M. BOYER, D. MARTINEZ, D. KIEFER, G. VUCOLO, A. WOLFE & S. SOCHER: Tumor secreting human TNF/cachectin induce cachexia in mice. Cell. 50: 555~559, 1987
16) STRASSMAN, G.; C. O. JACOB, R. EVANS, D. BEALL & M. FONG: Mechanisms of experimental cancer cachexia. Interaction between mononuclear phagocytes and colon 26 carcinoma and its relevance to IL-6 mediated cancer cachexia. J. Immunol. 148: 3674~3678, 1992
17) STRASSMAN, G.; Y. MASUI, R. CHIZZANITE & M. FONG: Mechanisms of experimental cancer cachexia-local involvement of IL-1 in colon-26 tumor. J. Immunol. 150: 2341~2345, 1993