

## Macrosphelide, a Novel Inhibitor of Cell-cell Adhesion Molecule

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

MASAHIKO HAYASHI, YONG-PIL KIM, HIDEKI HIRAOKA<sup>†</sup>, MASAHIKO NATORI<sup>†</sup>,  
SATOSHI TAKAMATSU, TOMOYA KAWAKUBO, ROKURO MASUMA,  
KANKI KOMIYAMA and SATOSHI ŌMURA\*

The Kitasato Institute,

5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan

<sup>†</sup>College of Agriculture and Veterinary Medicine, Nihon University,  
1866, Kameino, Fujisawa-shi, Kanagawa 252, Japan

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Potent anti-adherent activity was detected in fermentation extracts of microbial strain FO-5050. Active compounds designated macrosphelide A and B were isolated and the structure was determined to be 16-membered macrolide antibiotics possessing three ester bonds in the ring structure. Macrosphelide A dose-dependently inhibited the adhesion of HL-60 cells to LPS-activated HUVEC monolayer (IC<sub>50</sub>, 3.5 μM); macrosphelide B also inhibited HL-60 adhesion but to a lesser extent (IC<sub>50</sub>, 36 μM). Macrosphelide A did not show any antimicrobial and cytotoxic activities at the concentration of 1000 μg/ml and 100 μg/ml, respectively.

It has been reported that cell adhesion molecules play important roles in various types of pathological conditions, such as tumor<sup>1~3</sup>), allergy<sup>4~6</sup>) and inflammatory diseases<sup>7~9</sup>). Recent extensive study leads to the anti-adhesive concept and furthermore makes a promising strategy in the search for a new types of agents effective against tumor invasion and metastasis, and chronic inflammatory diseases, such as rheumatoid arthritis.

In the course of a screening program aimed at cell adhesion inhibitors obtained from microorganisms, macrosphelide was discovered in the fermentation broth of *Microsphaeropsis* sp. FO-5050, which was isolated from a soil sample. This paper deals with taxonomic studies of the producing strain, and the production, isolation and biological activity of new compounds, macrosphelide A and B.

#### Materials and Methods

##### General Experimental Procedures

Fungal strain FO-5050, isolated from a soil sample, was used for production of macrosphelide. DC-Alufolien Kieselgel 60 F<sub>254</sub> (Merck) was used for TLC analysis. HPLC was carried out using HITACHI D-2000 system and an ODS packed column (Senshu Pak Pegasil ODS, 5 μm, 20 × 250 mm).

##### Taxonomic Studies

Strain FO-5050 was originally isolated from a soil sample collected at Minami-izu-cho, Sizuoka Japan. For the identification of the fungus, potato-dextrose agar,

malt extract agar, yeast extract-soluble starch agar, potato carrot agar and corn meal agar were used. Morphological observation was done under a microscope (Olympus Vanox-S AH-2).

##### 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

Ten strains of established mammalian cells 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, respectively. To determine the cytotoxicity of macrosphelide, cells suspended in 200 μl of the medium were plated in a 96-well culture plate (Corning) and incubated for 24 hours at 37°C in a 5% CO<sub>2</sub>-95% air atmosphere. Five μl of medium containing a different concentration of macrosphelide was added to each well. After 72 hours of incubation, the cell growth was evaluated by the method of ALLEY *et al.*<sup>10</sup>.

##### Cell Adhesion Assay

HUVEC (human umbilical vein endothelial cells, 18~24 passages, Institute for Fermentation, Osaka) were stimulated with 50 ng/ml of LPS (*E. coli*, Difco) for 5

hours in 96-well plates. After 2 washes, macrospheptide A and B at various concentrations were added and incubated for 10 minutes. HL-60 cells (human leukemia) were prelabeled with CFSE (carboxyfluorescein diacetate succinimyl ester) by the method of BRONNER-FRASER<sup>11</sup>. Then, the CFSE-labeled HL-60 cells ( $1.0 \times 10^6/0.1$  ml/well) suspended in RPMI medium supplemented with 10% FCS were added and incubated for 30 minutes at 37°C. After removing the fluid and nonadherent cells by centrifugation, the number of attached cells (fluorescence intensity) to HUVEC was measured by Fluoroskan II (Dainippon Pharmaceutical Co., LTD., Osaka).

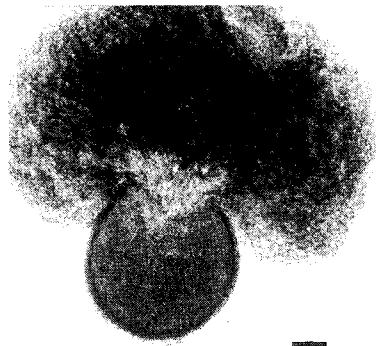
## Results and Discussion

### Taxonomy of Producing Organism

This strain, FO-5050, grew rapidly to form oyster white to brownish white colonies with a diameter of 70~90 mm after incubation for 14 days at 25°C. Pycnidia were moderate to abundant on the previous agar media. The color of the reverse side of the colonies was oyster white to yellowish brown. No soluble pigment was produced by the colonies.

Fig. 1. Photomicrograph of pycnidia of strain FO-5050 on potato-dextrose agar.

Bar represents 200  $\mu$ m.



When the strain FO-5050 was grown on potato-dextrose agar for 14 days at 25°C, the fungus produced rostrate pycnidia with loose hyphae. The pycnidia formed an ostiole and neck, and were 200~400  $\mu$ m in diameter as shown in Fig. 1. The conidia were oval to ellipsoidal and 4.5~5.5  $\times$  3.0~3.8  $\mu$ m in diameter as shown in Fig. 2. From these characteristics, the fungus was placed in the class Coelomycetes.

Also from the above characteristics, strain FO-5050 was further identified as a *Microsphaeropsis*<sup>12,13</sup>, and named *Microsphaeropsis* sp. FO-5050. This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology Japan, as FERM P-14776.

### Fermentation

The stock culture of the producing organism was inoculated into a test tube (20  $\times$  200 mm) containing 10 ml of a seed medium (glucose 2%, yeast extract 0.2%, polypeptone 0.5%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05%, KH<sub>2</sub>PO<sub>4</sub> 0.1% and agar 0.1% (adjusted to pH 6.0 before

Fig. 2. Photomicrograph of conidia of strain FO-5050 on potato-dextrose agar.

Bar represents 10  $\mu$ m.

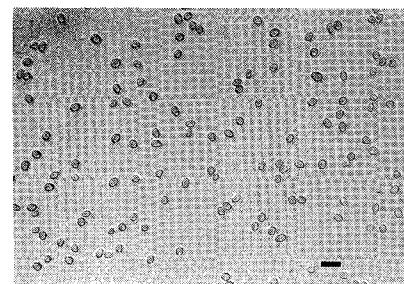


Fig. 3. Fermentation profile of macrospheptide production.

A) Anti-adhesive activity (%), B) Packed cell volume (%), C) pH.

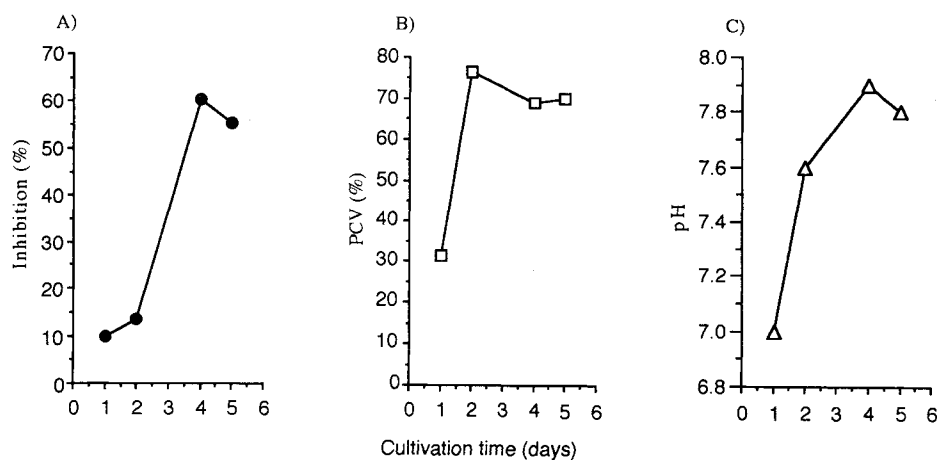
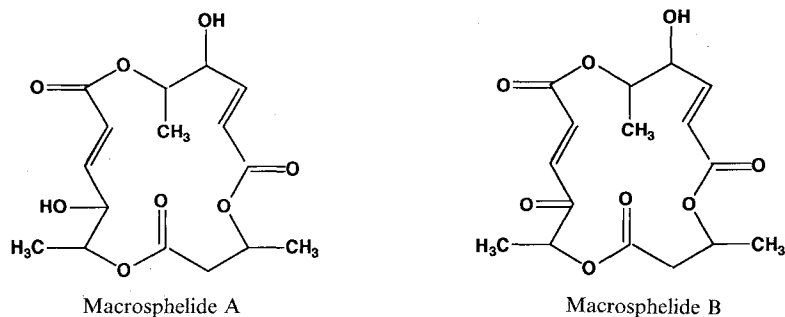


Fig. 4. Structure of macrosphelide.



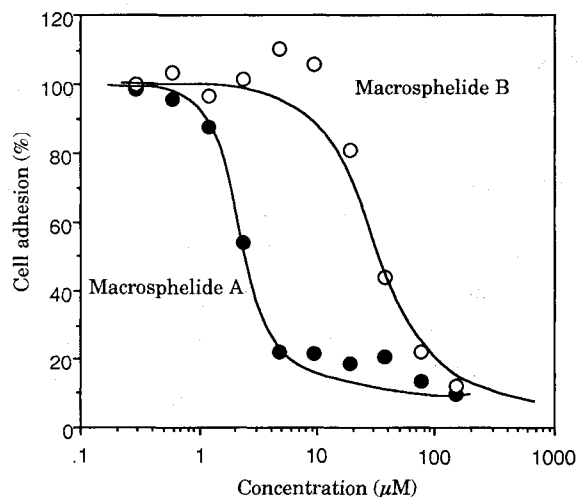
sterilization)). The test tube was incubated at 27°C for 72 hours on a reciprocal shaker. The resulting culture (3 × 10 ml) was transferred to a 500-ml Erlenmeyer flask containing 100 ml of production medium consisting of soluble starch 3.0%, glycerol 1.0%, S.B.M 2.0%, dried yeast 0.3%, KCl 0.3%, CaCO<sub>3</sub> 0.2%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05%, KH<sub>2</sub>PO<sub>4</sub> 0.05%, (adjusted to pH 6.5 with 6N HCl before sterilization) and the fermentation was carried out at 27°C for 5 days with an agitation rate of 200 rpm. A typical time course of the fermentation is shown in Fig. 3. Detection of the macrosphelide in the fermentation broth was followed by TLC analysis. After 4 days of fermentation, the amount of macrosphelide in the broth filtrate reached a maximum concentration (100 μg/ml).

#### Isolation and Purification

The culture broth (4 liter) of *Microsphaeropsis* sp. No. FO-5050 was filtrated, and the resulting supernatant was extracted with EtOAc (4 liter × 3) and then the organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The combined EtOAc layers were concentrated *in vacuo* to give a brown oil (6 g). The oily residue was applied to a silica column chromatography (E. Merck, Kieselgel 60, 70~230 mesh). The materials were eluted with a step wise gradient of CHCl<sub>3</sub>-CH<sub>3</sub>OH (100:0, 100:1, 50:1, 20:1, 10:1, each 500 ml). The active fractions (CHCl<sub>3</sub>-CH<sub>3</sub>OH, 50:1 and 10:1) were concentrated *in vacuo* to yield a powder material (CHCl<sub>3</sub>-CH<sub>3</sub>OH, 50:1, 180 mg, and CHCl<sub>3</sub>-CH<sub>3</sub>OH, 10:1, 530 mg, respectively).

The final purification of macrosphelide A (CHCl<sub>3</sub>-CH<sub>3</sub>OH, 10:1 fraction, 530 mg) was performed by using preparative HPLC fitted with ODS column (PEGASIL ODS (20 × 250 mm); Senshu Science Co. LTD., Japan; solvent, 40% aq. CH<sub>3</sub>CN: UV, 210 nm; flow rate, 8 ml/minute) to give a white powder. The CHCl<sub>3</sub>-CH<sub>3</sub>OH, 50:1 fraction (including macrosphelide B,

Fig. 5. Effect of macrosphelides on HL-60 cell-adhesion to HUVEC.



180 mg) was isolated by preparative TLC (Kieselgel 60 F<sub>254</sub>, Merck) developed with CHCl<sub>3</sub>-Acetone (8:2) to give a white powder (79.0 mg). The final purification of macrosphelide B was performed by using preparative HPLC in the same conditions as described above. Pure macrosphelide A (480 mg) and macrosphelide B (25 mg) were obtained as white powder, and the R<sub>f</sub> values on silica gel TLC (CHCl<sub>3</sub>-CH<sub>3</sub>OH, 9:1) were 0.5 and 0.8, respectively.

Structures of macrosphelide A and B are shown in Fig. 4. The studies on the structure determination of these metabolites will be reported in a separate paper.

#### Biological Activity

Macrosphelides were assayed in an adhesion assay system using HL-60 cells and HUVEC cells. As shown in Fig. 5, both macrosphelide A and B dose-dependently inhibited the adhesion of HL-60 cell to HUVEC stimulated with LPS. The IC<sub>50</sub> values of macrosphelide A and B were 3.5 μM and 36 μM, respectively.

Growth inhibitory activity of macrospinelides was examined against mammalian cells *in vitro*. Although the cells were continuously exposed to the compounds for 3 days, the growth of B16 melanoma, HeLa S3 carcinoma, P388 leukemia, L929 fibroblast, Shionogi carcinoma (SC-115), human prostate tumor (LNCap, PC-3), human leukemia (CEM, THP-1) and calf pulmonary artery endothelial cell (CPAE) were not affected at a concentration of 100  $\mu\text{g/ml}$  of macrospinelide A or B (data not shown).

No acute toxicity was observed when macrospinelide A was injected intraperitoneally into BDF1 mice at 200 mg/kg for 5 days (data not shown).

#### Other Biological Activities

The antimicrobial activity of macrospinelides was determined by the agar dilution method, and MICs of the antibiotics are listed in Table 1. Macrospinelide B was weakly active against bacteria, yeast and fungi, whereas macrospinelide A showed no activities at a concentration of 1000  $\mu\text{g/ml}$  against the microorganisms tested.

The carbohydrate antigen, Sialyl-Lewis<sup>X</sup> (SLe<sup>X</sup>), which is frequently present on human malignant cells including HL-60 human leukemia cell line<sup>14~16</sup>, is known to be a ligand for the cell adhesion molecule called ELAM-1 (endothelial cell-leukocyte adhesion molecule-1)<sup>14,17,18</sup>, which is expressed on cytokine- or LPS-activated human endothelial cells. ELAM-1 is one of the selectin family of adhesion molecules that contain a lectin motif to recognize SLe<sup>X</sup> antigen, and is peaked at 5 hours after stimulation on activated HUVEC<sup>18,19</sup>.

In our adhesion assay system, the adhesion of HL-60 cells to activated HUVEC also reached a maximum at 5 hours after LPS-stimulation and then reduced. In

addition, the expression of ELAM-1 at 5 hour on HUVEC was confirmed by flow cytometer (EPICS ELITE, Coulter, Tokyo) analysis using FITC-labeled anti-ELAM-1 antibody. Macrospinelide A inhibited the adhesion of HL-60 cells to LPS-activated HUVEC monolayer (IC<sub>50</sub>, 3.5  $\mu\text{M}$ ); macrospinelide B also inhibited HL-60 adhesion but to a lesser extent (IC<sub>50</sub>, 36  $\mu\text{M}$ ) in cell adhesion assay. Therefore, it seems most likely that macrospinelides inhibit the cell-cell adhesion by blocking the binding of SLe<sup>X</sup> to ELAM-1. Macrospinelide is a newly discovered anti-cell adhesion substance, which is, a low molecular weight, unique 16-membered macrolide antibiotics possessing three ester bonds. Therefore, it would be of interest to examine the mode of action of macrospinelide inhibiting cell-cell adhesion molecule and its availability for therapy against refractory disease such as tumor invasion and metastasis or chronic inflammatory disease.

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Table 1. Antimicrobial spectrum of macrospinelides.

Organisms	MIC ( $\mu\text{g/ml}$ )	
	macrospinelide A	macrospinelide B
<i>Staphylococcus aureus</i>	> 1000	250
<i>Micrococcus luteus</i>	> 1000	1000
<i>Bacillus subtilis</i>	> 1000	60
<i>Mycobacterium smegmatis</i>	> 1000	> 1000
<i>Escherichia coli</i>	> 1000	500
<i>Pseudomonas aeruginosa</i>	> 1000	> 1000
<i>Xanthomonas oryzae</i>	> 1000	> 1000
<i>Candida albicans</i>	> 1000	> 1000
<i>Saccharomyces sake</i>	> 1000	> 1000
<i>Aspergillus niger</i>	> 1000	> 1000
<i>Pyricularia oryzae</i>	> 1000	> 1000
<i>Mucor racemosus</i>	> 1000	> 1000
<i>Clostridium perfringens</i>	> 1000	> 1000
<i>Bacteroides fragilis</i>	> 1000	500
<i>Acholeplasma laidlawii</i>	> 1000	> 1000

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