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Adxanthromycins A and B, New Inhibitors of ICAM-1/LFA-1 Mediated Cell Adhesion Molecule from *Streptomyces* sp. NA-148

I. Taxonomy, Production, Isolation and Biological Activities

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Two new inhibitors of ICAM-1/LFA-1 mediated cell adhesion molecule, adxanthromycins A and B were isolated from the cultured broth of a streptomycete strain. The strain was identified as *Streptomyces* sp. NA-148 from its morphological and physiological characteristics. Adxanthromycins A and B inhibited the formation of the JY cell aggregates from 1.5 μg/ml, respectively, in a dose dependent manner. Components A and B also inhibited a human T cell leukemia cell line SKW-3 adhesion to soluble ICAM-1 in a dose-dependent manner with an IC_{50} of 18.8 μg/ml and 25.0 μg/ml, respectively.

Cell adhesion processes play roles in pathological conditions such as chronic inflammation and cancer metastases. Intercellular adhesion molecule-1 (ICAM-1: CD54) and lymphocyte function-associated antigen-1 (LFA-1: CD11a/CD18) are cell surface adhesion molecules that interact with one another to promote a number of cellular interactions, including antigen-specific T lymphocyte stimulation[1,2] and leukocyte adhesion to endothelium followed by emigration into sites of inflammation[3,4]. Treatment of animals with monoclonal antibodies directed against either ICAM-1 or LFA-1 resulted in inhibition of the inflammatory process. These results suggest that inhibitors of ICAM-1 or LFA-1 potentially have therapeutic value for inflammatory and immunological diseases[5~9]. In the course of our screening for inhibitors of ICAM-1/LFA-1 mediated cell adhesion molecule from microbial metabolites, we have found a new inhibitor, designated as adxanthromycin, in the cultured broth of strain *Streptomyces* sp. NA-148[10]. During the purification of adxanthromycin from the cultured broth, we discovered an additional new inhibitor designated as adxanthromycin B. In this paper we describe the taxonomical characterization, fermentation of the producing organism, isolation and biological activities of adxanthromycins A (**1**) and B (**2**) (Fig. 1). The physicochemical properties and structure elucidation is reported in an accompanying paper[11].

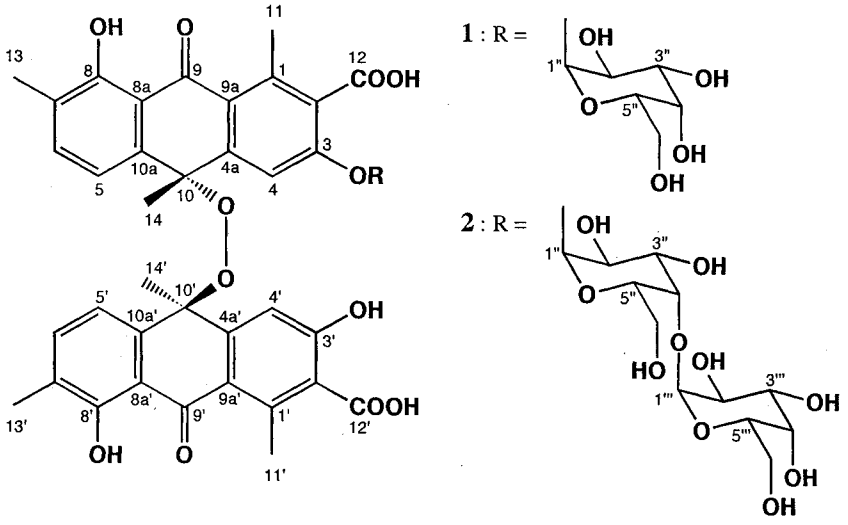
Materials and Methods

Microorganism and Cells

The adxanthromycin producing strain, NA-148, was isolated from a soil sample collected in Kanuma City, Tochigi Prefecture, Japan. This strain was maintained on Seino slant agar (soluble starch 1.0%, NZ amine type A 0.3%, yeast extract 0.1%, meat extract 0.1%, $CaCO_3$ 0.3%, agar 1.2%, pH 7.0). *Streptomyces* sp. NA-148 has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-6457.

JY Epstein-Barr virus-transformed B lymphoblastoid cells were provided by Dr. KO OKUMURA (Juntendo University School of Medicine, Tokyo, Japan). A human T cell leukemia cell line, SKW-3 was obtained from Hayashibara Biochemical Laboratories Inc., (Fujisaki Cell Center, Okayama, Japan).

Fig. 1. Structures of adxanthromycins A (**1**) and B (**2**).



Taxonomic Studies

Morphological observations were made with a scanning electron microscope (model Hitachi S-4100). Cultural and physiological characteristics of the strain NA-148 were examined by the methods of SHIRLING and GOTTLIEB[12]. For the evaluation of cultural characteristics, the strain was incubated in ISP media at 27°C for 2 to 3 weeks. The substrate and aerial mass color were assigned by Color Harmony Manual[13]. Carbohydrate utilization was examined by the method of PRIDHAM and GOTTLIEB[14]. The type of diaminopimelic acid isomers in the whole-cell was analyzed by the method of BECKER *et al.*[15].

A Homotypic JY Cell Aggregation Assay

JY cells were used for the homotypic cell aggregation assay[16]. The cells express both ICAM-1 and LFA-1 and easily aggregate each other after stimulation with phorbol myristate acetate (PMA). JY cells were suspended in RPMI 1640 medium (Nikken Bio Medical Laboratory, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS, Hy Clone, Utah) at 4×10^6/ml. Fifty μl of cell suspension were distributed in each well of a 96-well flat-bottom culture plate (Costar #3599) together with 50 μl of the test sample (diluted with the medium) followed by addition of 100 μl of PMA at a final concentration of 50 ng/ml. The plate was incubated at 37°C for 24 hours and cell aggregation was observed microscopically. Monoclonal antibody directed against ICAM-1 or LFA-1 was always included in the assay as a positive control and the inhibitory effect of test samples was compared with that of the antibodies.

JY Cell Cytotoxicity

The cytotoxicity of samples against JY cells was determined by a calorimetric analysis using WST-1[17]. Twenty μl of WST-1 was added to each well at the end of culture for evaluation of cytotoxicity of the test samples. After 4 hours incubation at 37°C, OD_{405} was measured and cytotoxicity of the test samples was calculated. The sample which inhibited JY cell aggregation without cytotoxicity was further evaluated for its activity in the cell adhesion assay.

An Adhesion Assay Using Soluble ICAM-1 and LFA-1 Positive SKW-3 Cells

An adhesion assay using soluble ICAM-1 and LFA-1 positive SKW-3 cells was carried out by the following method[18]. Soluble form of ICAM was prepared by fusing the first two Ig domains of ICAM-1 to the Fc portion of human IgG1[19]. Each well of 96-well microculture plate (Sumitomo Bakelite, Tokyo, Japan) was coated with 50 μl of soluble ICAM-1 [0.8 μg/ml in TSM buffer (25 mM Tris HCl, pH 7.8, 2 mM $MgCl_2$, 150 mM NaCl)] at 4°C over night and then was blocked with 2% bovine serum albumin (Fr. V, Sigma) in TSM buffer for 5 hours at 37°C. A human T cell leukemia cell line, SKW-3 cells, were used for the cell adhesion assay. The cells were suspended in RPMI 1640 medium supplemented with 10% of FBS at 1×10^7/ml

and labeled with 10 μM of BCECF-AM (3′-*O*-acetyl-2′,7′-bis (carboxyethyl)-4 or 5-carboxyfluorescein diacethoxy methyl ester, Dojindo Laboratories, Kumamoto, Japan) at 37°C for one hour. BCECF-labeled cells were then incubated with 20 ng/ml of PMA at 37°C for 30 minutes. The cells were washed once, resuspended in the medium at 8×10^6/ml and used for the adhesion assay. Fifty μl of BCECF labeled cells were distributed into each well of the plate together with 50 μl of adxanthromycins. After incubation at 37°C for 30 minutes, the wells were filled with the medium, and the plate was sealed with a plate seal (Sumitomo Bakelite, Tokyo, Japan) and left in an inverted position for 30 minutes at room temperature. The plate seal was then removed carefully and the non-adherent cells were aspirated off. The adherent cells were solubilized with 100 μl of 0.1% NP-40 and fluorescence intensity was measured with a multi-well fluorometer (Titertek Fluoroscan II, Flow Laboratories, Lugano, Switzerland) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Cytotoxicity Test Against SKW-3 Cells

SKW-3 cells were cultured in 96-well microplates at a concentration of 4×10^5/ml in the presence of adxanthromycins at 37°C for 20 hours. At the end of culture, 10 μl of alamar blue (BioSource International, CA) were added into each well and the plate was further incubated 4 hours. Fluorescence intensity was then measured with a multi-well fluorometer at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Cytotoxicity of adxanthromycins was determined from the comparison with the culture in the absence of adxanthromycins.

A Cell-free Adhesion Assay between ICAM-1 and LFA-1

The method was described elsewhere[19]. Briefly, microtiter plates were coated with soluble ICAM-1 (4 μg/ml in phosphate buffered saline) over night at 4°C (50 μl/well). After blacking with 2% BSA, the soluble LFA-1 (10 μg/ml) diluted with TSM buffer was added to the wells together with adxanthromycins for an hour at 25°C (50 μl of each/well). After washing with TSM buffer containing 0.05% Tween-20, the plates were incubated with biotinylated TS2/4 (non-neutralizing anti-LFA-1 mAb) followed by streptavidin-HRP (Amersham, Arlington Heights, IL, USA). The plates were washed and developed using ABTS substrate.

Results and Discussion

Taxonomy of The Producing Strain

The producing microorganism, strain NA-148, was isolated from a soil sample collected in Kanuma City, Tochigi Prefecture, Japan. The cultural characteristics of strain NA-148 grown on various media at 27°C for 21 days are shown in Table 1. The growth of aerial mycelium was good on every agar media employed. The strain formed straight sporophores on the aerial mycelium and the spores were cylindrical, 0.5~0.7×0.5~1.0 mm in size, having a

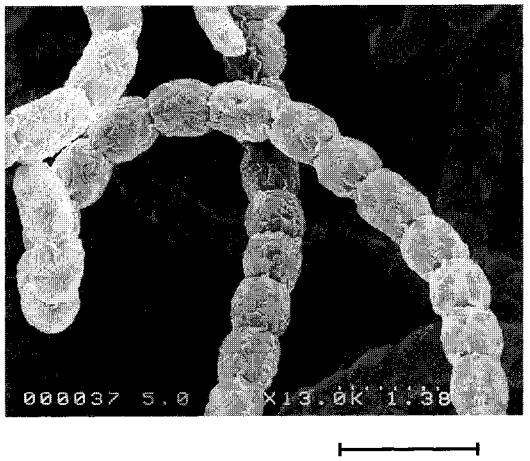
Table 1. Cultural characteristics of *Streptomyces* sp. NA-148.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Good	Abundant, light gray [2fe ~ 3fe]	Pale brown [3gc ~ 4gc]
Yeast extract-malt extract agar (ISP No. 2)	Good	Moderate, light gray [2fe ~ 3fe]	Brown [4pi]
Oatmeal agar (ISP No. 3)	Good	Thin, pale brown [3gc ~ 4gc]	Pale brown [3gc ~ 4gc]
Inorganic salts-starch agar (ISP No. 4)	Good	Moderate, white [a]	Yellowish brown [3ni]
Glycerol-asparagine agar (ISP No. 5)	Good	Moderate, light gray [2fe ~ 3fe]	Deep brown [4pl]
Tyrosine agar (ISP No. 7)	Good	Moderate, pale brown [3gc ~ 4gc]	Brown [4pi]
Glucose-asparagine agar	Good	Abundant, white [a]	None
Nutrient agar	Good	Thin, white [a]	None

smooth surface with some wrinkles on ISP agar medium (Fig. 2). The physiological properties of this strain are summarized in Table 2. Whole-cell hydrolysate analysis showed the presence of LL-diaminopimelic acid in the strain. On the basis of morphological and chemical characteristic, strain NA-148 was found to belong to the genus *Streptomyces*.

Fig. 2. The scanning electron micrograph of spore chains of *Streptomyces* sp. NA-148.

Bar represents 1.38 μm.



Fermentation

A loopful of a mature slant culture of *Streptomyces* sp. NA-148 was inoculated into a seed medium (10 ml) consisting of glycerol 2.0%, soybean powder 1.5%, $CaCO_3$ 0.3%, KH_2PO_4 0.01%, $Na_2HPO_4 \cdot 12H_2O$ 0.04%, $MgSO_4 \cdot 7H_2O$ 0.05% (pH 7.0) at 27°C for 3 days on a rotary shaker (230 rpm). The seed culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the same medium, then cultured at 27°C for 5 days on a rotary shaker (180 rpm). The whole culture was transferred into a 5-liter mini-jar fermentor containing 3 liters of the same medium and cultured at 27°C for 9 days (agitation; 250 rpm, aeration; 3 liters/minute). Production of the inhibitor in the fermentation broth was monitored by JY cell aggregation assay and an adhesion assay using soluble ICAM-1 and LFA-1 positive SKW-3 cells. The production of adxanthromycins began at day 3 and reached maximum at day 5.

Isolation

The procedure for the isolation of **1** and **2** is shown in Scheme 1. The fermentation broth was filtrated to give supernatant and mycelium cake. The mycelium cake was extracted with acetone. The extract was filtered and concentrated *in vacuo* to an aqueous solution. The solution was extracted twice with 2.5 liters of ethyl acetate. After evaporation, the crude powder (7.0 g) obtained was

Table 2. Physiological properties of *Streptomyces* sp. NA-148.

Spore chain morphology	Straight	Utilization of	
Spore surface	Smooth	D-Glucose	+
Aerial mass color	White	L-Arabinose	+
Melanin formation	+	D-Xylose	+
H_2S production	−	D-Fructose	−
Liquefaction of gelatin	+	Sucrose	+
Coagulation of milk	−	L-Rhamnose	+
Peptonization of milk	+	Raffinose	(−)
Cellulolytic activity	−	Inositol	+
Hydrolysis of starch	+	D-Mannitol	(−)
Temperature range for growth	20~37 °C	Control	(−)
NaCl tolerance	4~7 %		

+: Utilization, (−): probably no utilization, −: no utilization.

Scheme 1. Isolation procedure of adxanthromycins A (**1**) and B (**2**).

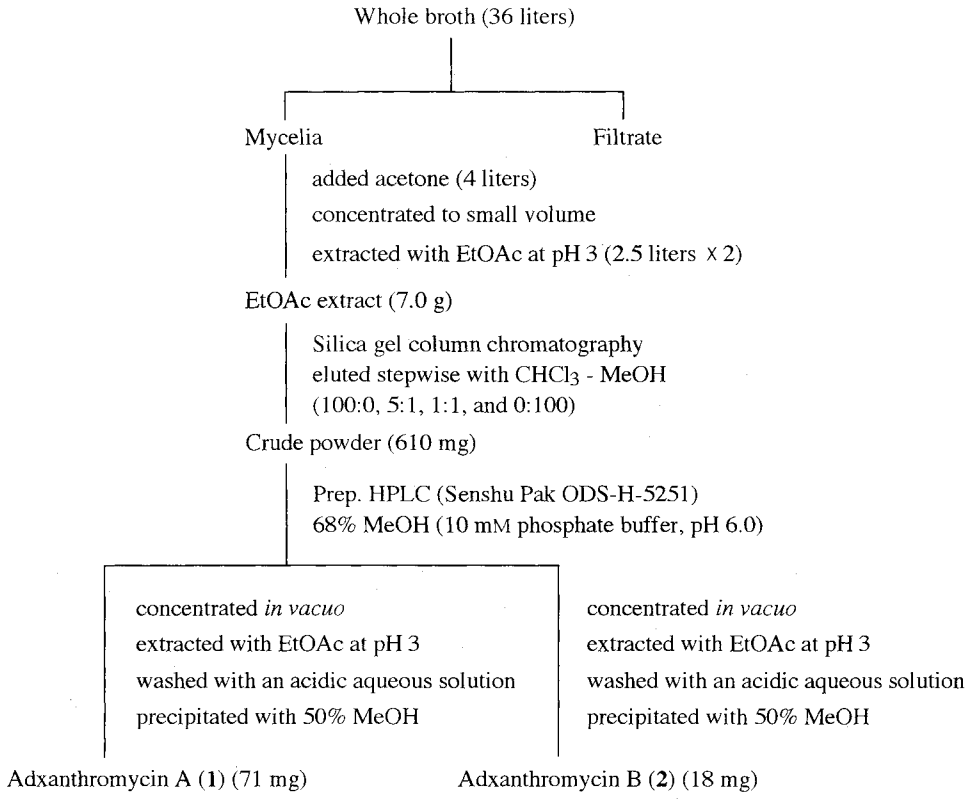
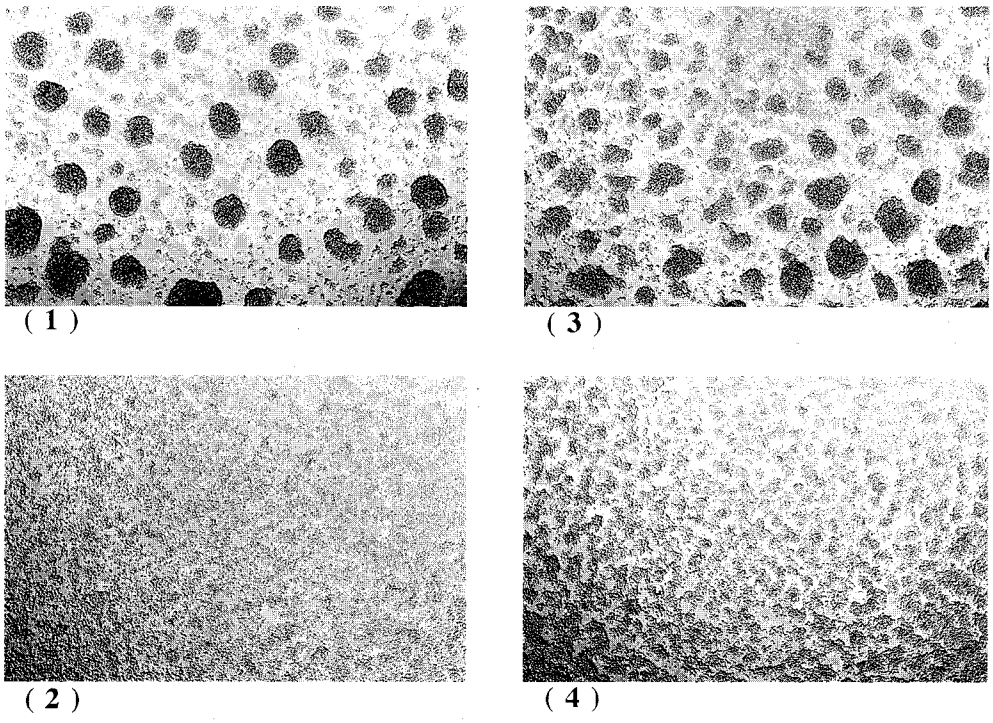


Fig. 3. Inhibition of homotypic aggregation of JY cells by adxanthromycin A (**1**).

(1) Negative control: PMA (+), (2) Positive control: TS1/22 (+) (anti-LFA-1 mAb, 10 μg/ml), (3) Adxanthromycin A (**1**): 1.56 μg/ml, (4) Adxanthromycin A (**1**): 6.25 μg/ml.



applied to a column of silicagel 60 with a gradient of chloroform/methanol. The crude material (610 mg) obtained from this chromatography was subjected to preparative HPLC analysis (mobile phase; methanol-10 mM phosphate buffer 68:32, flow rate; 8 ml/minute) using an ODS column (Senshu Pak ODS-H-5251, 20 mm i.d.×250 mm). Active fractions were concentrated *in vacuo*. The residue was extracted with ethyl acetate at pH 3. The extract was concentrated *in vacuo* to obtain the powder of **1** and **2**. Each powder was washed with an acidic aqueous solution and then with 50% aqueous methanol to isolate **1** and **2** as a pale yellowish powder.

Biological Activities

Compounds **1** and **2** inhibited homotypic aggregation of JY cells from 1.5 μg/ml in a dose dependent manner. A complete inhibition was observed at 6.25 μg/ml (Fig. 3). Potency of inhibitory activities of compounds **1** and **2** in the aggregation assay was almost the same. The toxicity (IC_{50}) of **1** and **2** against JY cell was 15.2 μg/ml. Compounds **1** and **2** also inhibited SKW-3 adhesion to soluble ICAM-1 in a dose-dependent manner with an IC_{50} of 18.8 μg/ml and 25.0 μg/ml, respectively. On the other hand, the cell toxicity (IC_{50}) of adxanthromycins against SKW-3 was 110.0 μg/ml. The inhibitory activity of the compounds in the cell-free receptor binding assay was examined in the manner reported previously[19]. Adxanthromycins showed weak inhibition in the assay with an IC_{50} of 760 μg/ml in the cell-free system.

MUSZA *et al.* have reported novel inhibitors of ICAM-1/LFA-1 mediated adhesion molecule, seco-limonoids[20] and cucurbitacin E[21] isolated from the root of *Trichilia rubra* (Meliaceae), and from the stem and leaves of *Conobea scoparioides* (Scrophulariaceae), respectively. Compounds **1** and **2** are the first example of inhibitors of ICAM-1/LFA-1 mediated adhesion molecule isolated from microbial sources.

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