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Characterization of *Mycotypha* Metabolites Found to be Inhibitors of Cell Adhesion Molecules

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Three inhibitors of cell adhesion based on LFA-1/ICAM-1 were isolated from the cultured broth of the fungal strain *Mycotypha* sp. UMF-006. These compounds were identified by spectroscopy to be cytochalasin E (**1**), 5,6-dehydro-7-hydroxy derivative of cytochalasin E (**2**) and $\Delta^{6,12}$-isomer of **2** (**3**). All these components inhibited adhesion of HL-60 cells to CHO-ICAM-1 cells at IC_{50} values of 30 μg/ml for **1**, 75 μg/ml for **2**, and 90 μg/ml for **3**.

Cell adhesion molecules (CAMs) are of fundamental importance in the regulation of immunity, inflammation, tissue remodeling, and embryonic development. For example, leukocyte adherence to the endothelial cell is an essential event in the process of inflammation and immune recognition[1,2], and the extracellular interactions between specific CAMs expressed on the endothelium and leukocytes will mediate leukocyte entry into tissues, T-cell proliferation, and antigen presentation[3~5]. An agent that could inhibit leukocyte adhesion and transmigration would represent a novel mechanism of action as an immunosuppressive and anti-inflammatory drug. The major adhesion event for lymphocyte extravasation from the blood stream into the tissue sites is the protein-protein interaction of the adhesion molecules lymphocyte function-associated molecule 1 (LFA-1, CD11a/CD18, β2 integrin) and its endothelial counter receptor intercellular adhesion molecule 1 (ICAM-1, CD54). Clinical trials using monoclonal antibodies to ICAM-1 have been shown to inhibit lymphocyte transendothelial migration and have yielded very promising results in the treatment of rheumatoid arthritis and organ transplantation[6,7]. Therefore, specific inhibitors of integrin-mediated cell adhesion with a small molecule may have therapeutic potential as anti-inflammatory, immunosuppressive and antimetastatic agents.

In this study, we used a combination of two different methods to screen for inhibitors of cell adhesion between LFA-1 and ICAM-1. The primary method used was the cell aggregation inhibitory assay based on the myelomonocytic cell line HL-60, and the second used was the XTT cell proliferation assay. Secondary screening was performed based upon the direct cell-cell adhesion assay through contact between LFA-1 of HL-60 cell and ICAM-1 of Chinese hamster ovary cell lines (CHO).

Cultures of different microbial isolates from soil samples were screened, and one fungal isolate (*Mycotypha* sp. UMF-006) was found to produce cell adhesion inhibitors. In this study, we describe the cultivation of the producing strain, and isolation and structure of the CAM inhibitors (cytochalasin E and its derivatives) and their biological properties.

Materials and Methods

Taxonomic Studies

A fungal strain, UMF-006 was originally isolated from a

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soil sample collected in Oxford, Mississippi, U.S.A., by spread plating diluted soil samples on potato dextrose agar plates. Morphological observation and identification were performed using a phase contrast microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY) and identification guides[8,9].

Growth Conditions and Isolation of the Active Compounds

The fungal strain UMF-006 was cultivated in test tubes (i.d. 25×200 mm) containing 10 ml of potato dextrose agar medium (potato; infusion 0.4%; dextrose 2.0%; agar 0.1%) and placed on a rotary shaker (Model 3529; Lab-Line, Melrose, IL) held at 27°C with shaking at 220 rpm for 3 days. The tube cultures are used as inoculation (1%) for 500-ml Erlenmeyer flasks (40 total) containing 100 ml of the same medium and at 27°C for 6 days on a rotary shaker (210 rpm). The total cultured broth (4 liters) was filtered and the filtrate was extracted with ethyl acetate (EtOAc). The EtOAc extract was subjected to silica gel chromatography with $CHCl_3$-MeOH as a developing solvent to obtain a crude fraction containing **2** and **3**. The mycelial portion was extracted successively with methanol (MeOH) and EtOAc. The EtOAc mycelium extract was then subjected to silica gel chromatography and eluted with $CHCl_3$-MeOH. Final purification of each compound (**1**~**3**) by use of preparative HPLC (μBonda pack, Waters, Milford, MA; 80% MeOH-H_2O; Detect. 210 nm) yielded **2** (6.8 mg) and **3** (6.5 mg) from the water layer extract, and **1** (9.9 mg) from the mycelium extract.

Cell Culture and Antibodies

The cell lines used in this study were HL-60 (ATCC), CHO-ICAM-1 (ATCC), and CHO-K1 (ATCC), and each cell line was cultured in RPMI-1640 medium (GIBCO, Rockville, MD) containing 10% fetal bovine serum (HyClone, Logan, UT) and 60 μg/ml amikacin (Sigma, St. Louis, MO) at 37°C in air containing 5% CO_2. HL-60 cells were subcultured at a density of 2×10^5 cells/ml and treated with retinoic acid (1 mM, Sigma).

The following antibodies purchased from Santa Cruz Biotech, Inc. (Santa Cruz, CA) were used for flow cytometric analysis: monoclonal anti-LFA-1 and anti-ICAM-1.

Flow Cytometric Analysis

Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG was used as a second antibody. Phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free, pH 7.4) containing BSA 1% and sodium azide (NaN_3) 0.1% was used as a staining medium. PBS(−) buffer containing BSA 0.1% and NaN_3 0.05% was used as a washing solution. Cells were stained at 4°C in the dark and analyzed by FACSCalibur (Becton Dickinson, San Diego, CA). Fluorescence intensity was determined on 10,000 cells from each sample.

Cell Aggregation Assay[10]

The promyelocytic cell line HL-60 was suspended at a density of 1×10^6 cells/ml. Cell suspension was added to wells of 96-well plate (150 μl/well). After incubation with test sample for 10 minutes, phorbol 12-myristate 13-acetate (PMA, Sigma, 10 ng/ml, final) was added. Microplates were placed in a CO_2 incubator (air containing 5% CO_2 at 37°C) and aggregation of the cells was observed at 16 hours after the PMA addition. The cell aggregation inhibitor cytochalasin B, anti-LFA-1, and anti-ICAM-1 monoclonal antibodies were used as positive controls.

XTT Assay for Cytotoxicity of HL-60 Cells

Following the cell aggregation assay, the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] assay was performed using the method described by SCUDIERO *et al.*[11]. Briefly, 25 μl of XTT-phenazine methosulfate (PMS) solution (1 mg/ml XTT solution supplemented by 25 μM of PMS) were added to the wells containing cells. After incubating for 4 hours at 37°C, absorbance (450 nm) was measured by using a microplate reader (EL312e; Bio-Tek Instruments, Winooski, VT) with reference absorbance at 630 nm.

Cell Adhesion Assay

HL-60 cells that express LFA-1 were stained with a CFSE (carboxyfluorescein diacetate succinimyl ester; Molecular Probes, Eugene, OR)[12]. CFSE-labeled HL-60 cells and potential inhibitors were added to the wells of 96-well microtiter plates which contained confluent monolayers of CHO-ICAM-1 cells expressing high levels of intercellular adhesion molecule-1 (ICAM-1)[13]. Then, 100 ng/ml PMA was added to stimulate the HL-60 cells to convert LFA-1 to its high affinity binding state[14]. The cultures were incubated for 45 minutes at 37°C and then prewarmed medium added carefully to completely fill the wells. The plates were sealed with 96-well plates-format storage mats. After fixing a lid with binder clips, the microplate was inverted and held upside down for 30 minutes at room temperature in the dark. Nonadherent HL-60 cells were washed off with PBS (−) buffer. The remaining cells were solubilized with 1% Triton X-100 (Sigma) in PBS (−) buffer and fluorescence was

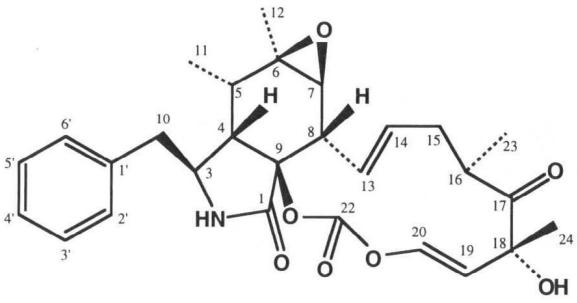
quantitated using a CytoFluor 2350, Fluorescence Measurement System (Millipore, Milford. MA) with an excitation wavelength of 496 nm and emission at 519 nm. Anti-LFA-1 and anti-ICAM-1 monoclonal antibodies were used as positive controls.

Results

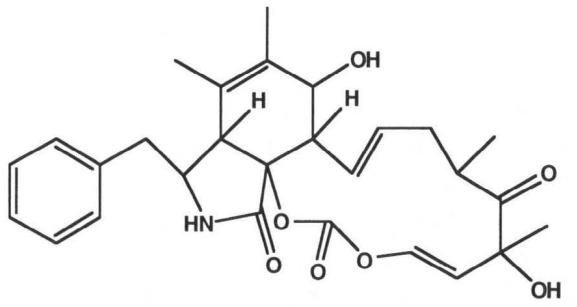
Strain Taxonomy

The producing strain UMF-006 was determined to be a *Mycotypha* species (Figure 2) from morphological studies. The very distinct morphology of the heads of the conidiophores and arrengement of conidia of UMF-006 aided in the identification process[8,9].

Fig. 1. Structures of **1**~**3**.



1



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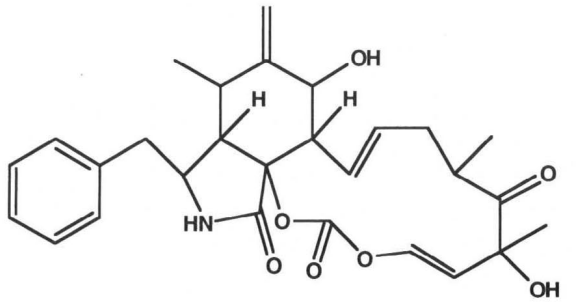
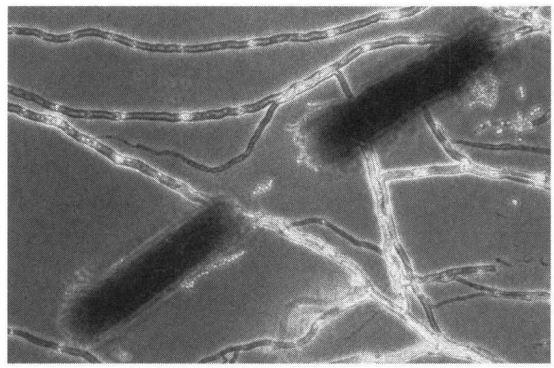


3

Expression of Cell Adhesion Molecule on HL-60 Cells and CHO-ICAM-1 Cells

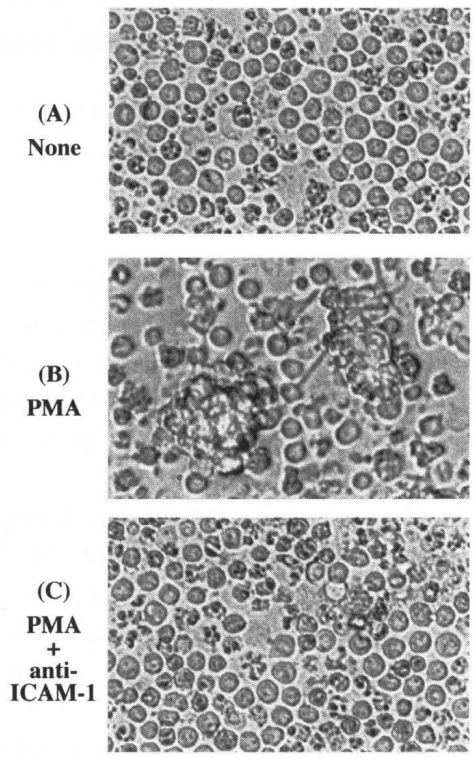
As shown in Figure 3, PMA induced cellular aggregation

Fig. 2. Photograph of *Mycotypha* sp. UMF-006 grown on potato-dextrose agar.



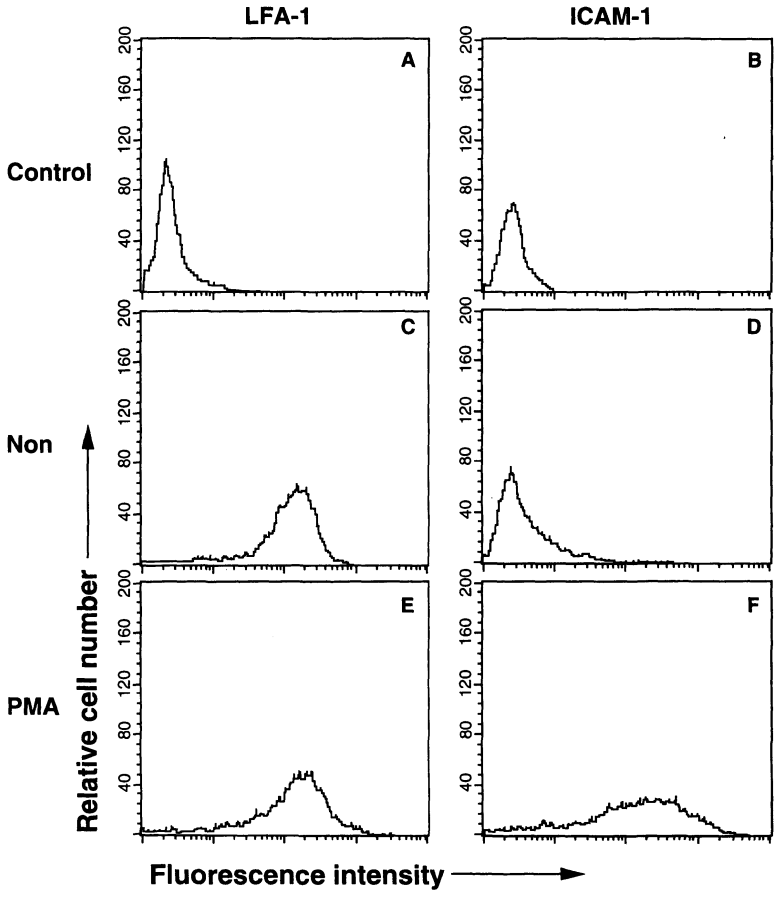
Magnification=200×.

Fig. 3. PMA-induced LFA-1/ICAM-1-dependent aggregation of HL-60 cells.



Cells are non-stimulated (A, None) or stimulated after 16 hours with 10 ng/ml (B, PMA) in the presence of 1 μg/ml anti-LFA-1 anybody (C, PMA+anti-ICAM-1). Micrographs were created using a Nikon Eclipse, TE 300 microscope. Total magnification is 400×.

Fig. 4. Expression of LFA-1 and ICAM-1 on HL-60 cells non-stimulated (C and D, Non) or stimulated after 16 hours with 10 ng/ml (E and F, PMA).



The control profiles used a second antibody without inclusion a first antibody (A and B, control).

of HL-60 cells within 16 hours after stimulation (Figure 3B). The cellular aggregation was completely inhibited by anti-LFA-1 and anti-ICAM-1 antibodies at 1 μg/ml (Figure 3C shows only an effect of an anti-ICAM-1 antibody), indicating that the aggregation was LFA-1/ICAM-1-dependent.

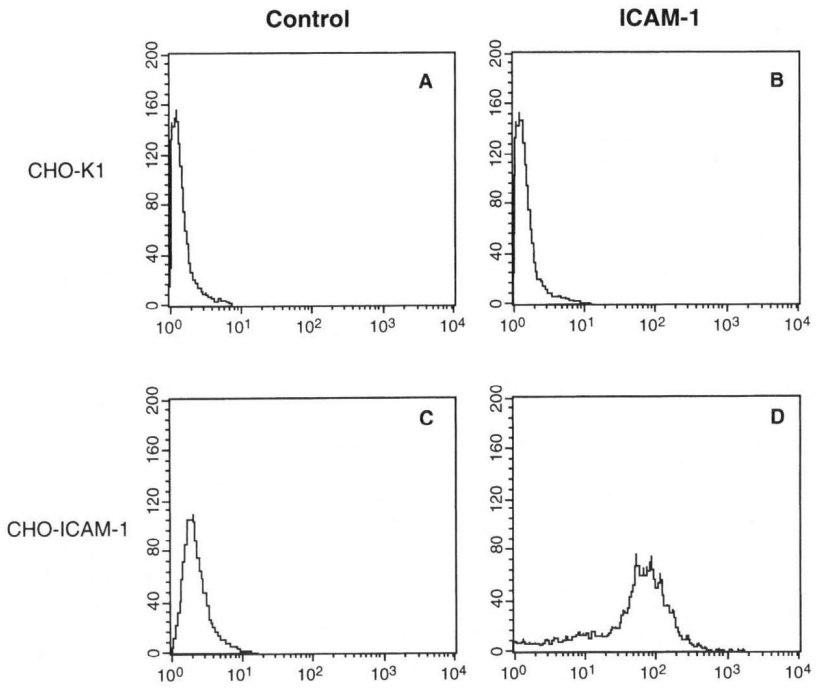
In order to confirm presence of cell adhesion molecules, flow cytometric analyses were performed. As shown in Figure 4, LFA-1 is ubiquitously expressed on HL-60 cells before and after stimulation with PMA (Figures 4C and E). On the other hand, ICAM-1 is only expressed after stimulation (Figure 4F).

For the cell-cell adhesion assay using two different cell lines, HL-60 cells does not express ICAM-1 within 2 hours after stimulation. Therefore, expression of the counter receptor of LFA-1, ICAM-1 is necessary on another cell line CHO-ICAM-1. CHO-ICAM-1 cells were originally established by HASLER *et al.*[13] to be stable transformants expressing high levels of human ICAM-1 as illustrated in Figure 5D. In our study, HL-60 cells attached to CHO-ICAM-1 cells after 30 minutes of PMA stimulation (Figure 6B), whereas adhesion of HL-60 cells to CHO-ICAM-1 was not observed in the absence of PMA (Figure 6A). Therefore, activation of the LFA-1 high affinity binding site is necessary for adhesion of HL-60 cells into CHO-ICAM-1 cells[14].

Isolation and Structure Elucidation

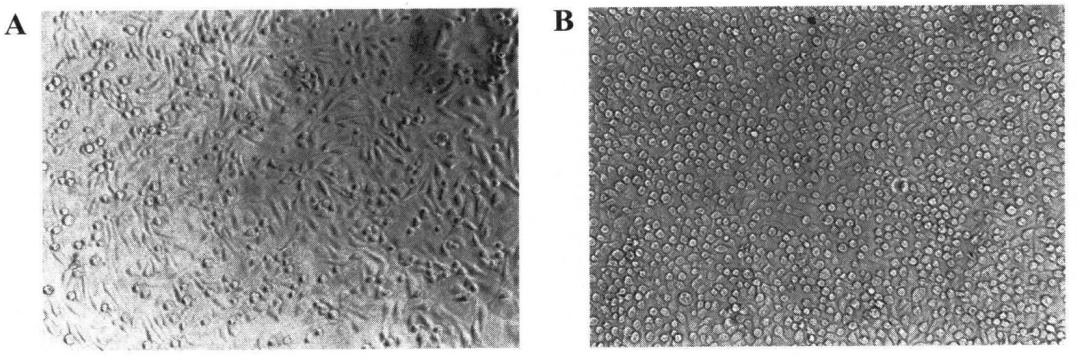
Bioassay-guided isolation led to the discovery of three biologically active compounds. The main compound (**1**) was identified as cytochalasin E based upon mass, IR, UV, ^{1}H- and ^{13}C-NMR spectroscopic data (Tables 1 and 2). The other two minor components were identified as 5,6-

Fig. 5. Expression of ICAM-1 on CHO-ICAM-1 cells.



CHO-ICAM-1 cells express high levels of ICAM-1 (D). ICAM-1 was not detected on CHO-K1 cells (B). The control profiles using a second antibody without a first antibody are also shown (A and C).

Fig. 6. PMA stimulation is necessary for adhesion of HL-60 cells to CHO-ICAM-1 monolayers.



Unstimulated HL-60 cells bind little to CHO-ICAM-1 cells (A). Significant adherences of HL-60 cells stimulated with 100 ng/ml PMA were observed (B).

dehydro-7-hydroxy derivative of **1** (**2**)[15] and $\Delta^{6,12}$-isomer of **2** (**3**)[16].

Biological Activity of Compounds **1**~**3**

The inhibitory effect of cytochalasin E (**1**) and its derivatives (**2**, **3**) on cell aggregation, cell adhesion, and cell proliferation are shown in Table 3. Compound **1** was a potent inhibitor on cell aggregation and cell adhesion assay compared to **2** and **3** without cytotoxicity, at least up to 50 μg/ml.

Table 1. ^{1}H-NMR (500 MHz) chemical shifts of **1**~**3** in $CDCl_3$.

H	1	2	3
3	3.78 (1H, br.s)	3.84 (1H, t)	3.45 (1H, t)
4	3.03 (1H, dd, 4.4, 2.4)	4.31 (1H, br.s)	3.03 (1H, br.t)
5	2.27 (1H, m)	–	3.33 (1H, br.s)
7	2.64 (1H, m)	4.50 (1H, br.t)	3.73 (1H, d, 12.0)
8	2.64 (1H, m)	3.47 (1H, t, 10.0)	2.91 (1H, t, 10.0)
10a	2.90 (1H, dd, 13.5, 5.0)	2.99 (1H, m)	2.94 (1H, m)
10b	2.72 (1H, dd, 13.5, 7.0)	3.05 (1H, m)	2.72 (1H, m)
11	1.07 (3H, d, 8.0)	1.48 (3H, s)	1.04 (3H, d, 7.0)
12a	1.26 (3H, s)	1.85 (3H, s)	5.15 (1H, br.s)
12b	–	–	5.35 (1H, br.s)
13	5.89 (1H, dd, 15.0, 8.0)	6.91 (1H, dd, 15.5, 10.0)	5.60 (1H, dd, 15.5, 10.0)
14	5.22 (1H, m)	5.51 (1H, ddd, 14.5, 11.0, 3.5)	5.29 (1H, m)
15a	2.64 (1H, m)	2.05 (1H, br.d, 15.0)	2.52 (1H, m)
15b	2.15 (1H, br.d)	2.83 (1H, d, 10.0)	2.13 (1H, br.d, 12.0)
16	2.90 (1H, m)	2.99 (1H, m)	3.05 (1H, m)
19	5.59 (1H, d, 12.0)	6.07 (1H, d, 11.5)	5.80 (1H, d, 11.5)
20	6.43 (1H, d, 12.0)	7.20 (1H, m)	6.32 (1H, d, 11.5)
23	1.14 (3H, d, 7.0)	1.00 (3H, d, 5.0)	1.11 (3H, d, 5.0)
24	1.46 (3H, s)	1.57 (3H, s)	1.47 (3H, s)
2', 6'	7.16 (2H, d, 7.2)	7.26 (2H, d, 7.0)	7.16 (2H, br.d, 7.0)
3', 5'	7.32 (2H, t, 7.2)	7.25 (2H, m)	7.32 (2H, t, 7.5)
4'	7.27 (1H, t, 7.2)	7.20 (1H, m)	7.25 (1H, m)

(Multiplicity, *J* value in Hz)

Discussion

In this study, we developed a LFA-1/ICAM-1 dependent cell-cell adhesion assay using HL-60 cells and CHO-ICAM-1 cells. As CHO-ICAM-1 cells ubiquitously express high levels of human ICAM-1, induction of ICAM-1 for several hours with cytokines is not required[17]. Therefore, CHO-ICAM-1 cells are convenient to screen for cell adhesion inhibitors alternative to the use of soluble ICAM-1 on cell free systems. In the process of searching for cell adhesion inhibitors of microbial origin, we discovered cytochalasin E derivatives in the cultured filtrate of *Mycotypha* sp. UMF-06 to be LFA-1/ICAM-1 adhesion inhibitors (Figure 1). Cytochalasin E (**1**) is a well known fungal metabolite affecting cytoskeletal formation[18] and lipid droplet formation for atherosclerosis[19]. Compound (**1**) and its derivatives showed inhibition of cell aggregation of HL-60 cells without cytotoxicity, at least up to 50 μg/ml. Of the three inhibitors identified, **3** was the most selective inhibitor of cell aggregation. On the other hand, **1** was a potent inhibitor of cell adhesion of HL-60 cells to CHO-ICAM-1 cells. Compound (**2**) has previously been identified as cytochalasin K isolated from *Aspergillus clavatus*[15]. Compound (**3**), a $\Delta^{6,12}$-isomer of **2** with exocyclic methylene group, was suggested to be a decomposition product of cytochalasin E (**1**) by KAJIMITO *et al.*[16]. However, an acid-catalyzed rearrangement of **1** yields **2** and **3**[15]. Therefore, **3** may actually be an artificial product of **1**. In any case, it is undeniable that **3** appears to be an artificial natural product. The structure determination of **2** and **3** were reported on the basis of ^{1}H-NMR data and chemical conversion[15,16]. Since ^{13}C-NMR chemical shifts of **2** and **3** have not been reported, the complete assignments of chemical shifts of **2** and **3** were shown in Table 2. Our study is the first to report of the isolation of **2** and **3** as natural products produced by a fungal isolate and include biological activities. It may be of particular interest to consider drug design based upon a common structural unit perhydroisoindole of cytochalasin E (**1**) and its derivatives **2** and **3**. Some inhibitors of ICAM-1/LFA-1 mediated cell adhesion molecules derived from plants have been reported[20~22].

Recently, adoxanthromycins A and B of microbial origin were reported to be primary inhibitors of ICAM-1/LFA-1 mediated cell adhesion[23]. These compounds were found to

inhibit the JY-cell aggregation and the adhesion of SKW-3 cells to soluble ICAM-1. Another group of specific cell adhesion inhibitors, macrosphelides[24] were reported to inhibit E-selectin/sialyl Lewis^x-mediated cell adhesion[25]. These examples indicate that a variety of specific inhibitors of cell adhesion molecules may be further evaluated as leads for anti-inflammatory and anti-metastatic agents.

Table 2. ^{13}C-NMR (125 MHz) chemical shifts of **1**~**3** in $CDCl_3$.

C	1	M	2	M	3	M
1	170.5	s	170.1	s	172.5	s
3	53.9	d	59.2	d	54.7	d
4	47.8	d	48.5	d	47.5	d
5	36.0	d	127.4	s	33.5	d
6	57.5	s	136.8	s	151.5	s
7	60.8	d	70.2	d	71.6	d
8	45.9	d	50.1	d	49.7	d
9	87.4	s	86.4	s	88.6	s
10	44.7	t	44.3	t	43.8	t
11	13.3	q	17.9	q	15.0	q
12	19.9	q	14.2	q	114.6	t
13	128.7	d	131.9	d	130.4	d
14	131.6	d	133.7	d	133.8	d
15	39.3	t	39.2	t	40.8	t
16	41.0	d	41.1	d	42.4	d
17	212.0	s	211.7	s	213.7	s
18	77.6	s	77.5	s	79.0	s
19	120.5	d	120.6	d	123.0	d
20	142.3	d	142.6	d	142.7	d
22	149.5	s	149.2	s	151.3	s
23	20.3	q	20.4	q	20.8	q
24	24.5	q	24.8	q	24.9	q
1'	136.1	s	136.8	s	138.0	s
2', 6'	129.9	d	129.5	d	131.8	d
3', 5'	128.7	d	129.6	d	129.9	d
4'	127.5	d	127.4	d	128.3	d

M, Multiplicity

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Table 3. Effect of **1**~**3** on cell aggregation, cell proliferation, and cell adhesion.

	Cell Aggregation (A)	Cell Proliferation (B)		Cell Adhesion (C)	
	MIC (μg/ml)	IC_{50} (μg/ml)	S.I. (B)/(A)	IC_{50} (μg/ml)	S.I. (B)/(C)
1.	0.09	> 50	> 555.6	30	> 1.7
2.	2.31	60	26.0	75	0.8
3.	0.77	> 62.5	>81.2	90	> 0.69
Cyt. B	3.5	> 31.3	>8.9	7.5	> 4.2

MIC, Minimum Inhibitory Concentration; S.I., Specific Index

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