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New Cell-cell Adhesion Inhibitors from *Streptomyces* sp. UMA-044

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Three compounds, NP25301 (**1**), NP25302 (**2**) and bohemamine (**3**), inhibitors of cell adhesion based on LFA-1/ICAM-1, were isolated from the cultured broth of the strain *Streptomyces* sp. UMA-044. New compounds **1** and **2** were identified as 2-(3′-carbamoylphenoxy)acrylic acid methyl ester and deoxybohemamine, respectively, based on spectroscopic analyses. Compounds **1**~**3** inhibited adhesion of HL-60 cells to CHO-ICAM-1 cells at IC_{50} values of 29.5 μg/ml for **1**, 24.3 μg/ml for **2**, and 27.2 μg/ml for **3**.

Intercellular cell adhesion molecule-1 (ICAM-1, CD54) is a cell adhesion molecule that plays an important role in inflammatory processes and in a T-cell mediated host defence systems. Lymphocyte function-associated molecule 1 (LFA-1, CD11a/CD18, β2 integrin) is a leukocyte cell surface glycoprotein, a counter receptor of ICAM-1 that promotes intercellular adhesion in immunological and inflammatory reactions[1~5]. The LFA-1/ICAM-1 mediated cell adhesion pathway is important in the progression of inflammatory responses *in vivo*, including rheumatoid arthritis and ischemia-reperfusin injury. Therefore, inhibitors of cell adhesion based on LFA-1/ICAM-1 could be novel therapeutic drugs for inflammatory diseases. In order to screen for inhibitors of cell adhesion based on LFA-1/ICAM-1, a combination of the cell aggregation inhibitory assay on the myelomonocytic cell line HL-60 and a direct cell-cell adhesion assay between HL-60 cell and Chinese hamster ovary cell lines expressing ICAM-1 (CHO-ICAM-1) was performed for primary and for secondary assay, respectively. We have reported discovery of cytochalasin E derivatives in the cultured filtrate of *Mycotypha* sp. UMF-06 to be LFA-1/ICAM-1 adhesion inhibitors[6].

In our continuing search for novel inhibitors of cell-cell adhesion molecules based on LFA-1/ICAM-1, NP25301 (**1**) and NP25302 (**2**) along with the known compound bohemamine (**3**)[7,8] were isolated from a fermentation broth of *Streptomyces* sp. UMA-044, which had been isolated from a sediment collected in a catfish pond, Stoneville, Mississippi U.S.A. (Fig. 1). In this study, we describe the cultivation of the producing strain, and the isolation and structure of the inhibitors of cell adhesion molecule and their biological properties.

Materials and Methods

General Experimental Procedures

Specific rotation was obtained on an AutoPol IV automatic polarimeter. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) and 2D NMR were recorded on a Bruker Avance DRX 500. HR-ESI-MS were measured on a Bruker-Magnex BioAPEX 30es ion cyclotron high-resolution HPLC-FT spectrometer by direct injection into an electrospray interface. IR was recorded on an ATI Mattson Genesis Series FT-IR. TLC were performed on aluminum backing silica gel 60 F_{254} (EM Science) or reversed-phase $KC_{18}F$, Silica gel 60 (Whatman). Silica gel (230~400 mesh, Natland International Corporation) was used for column chromatography.

Taxonomic Studies

The bacterial strain, UMA-044, was originally isolated from a sediment sample collected in a catfish pond,

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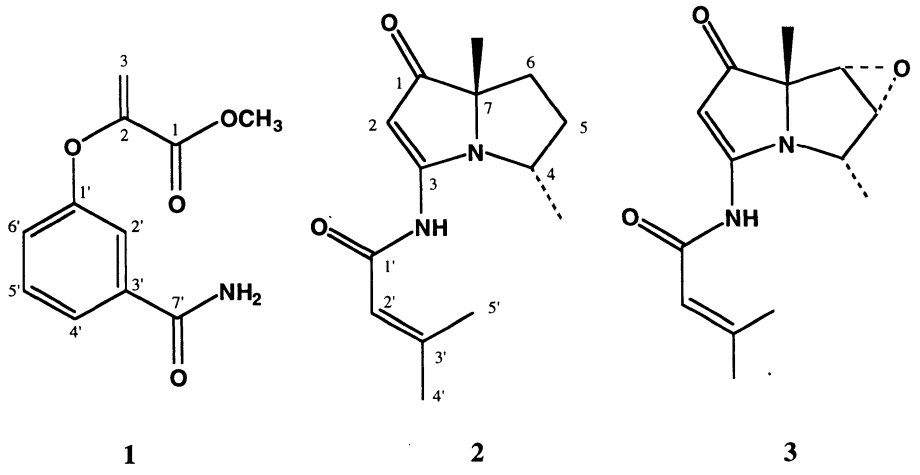
Fig. 1. Structures of **1**~**3** produced by *Streptomyces* sp. UMA-044.



1 **2** **3**

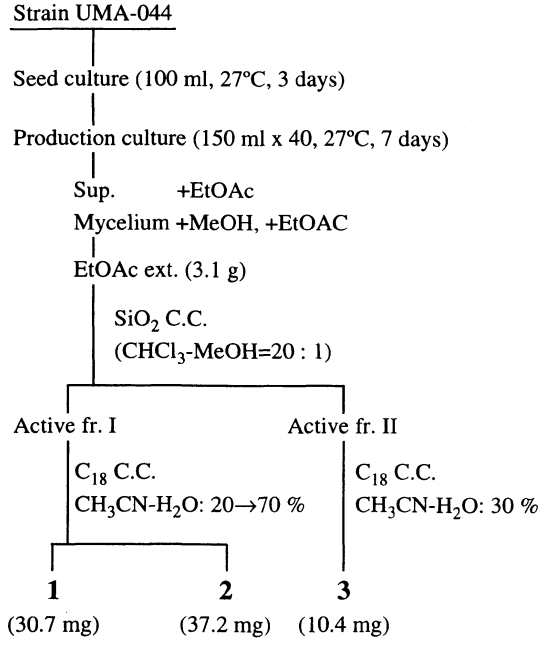
Stoneville, Mississippi, U.S.A. The basic morphological properties were observed using a phase contrast microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY). The organism was identified based on its 16S RNA gene sequence (the MicroSeq®; Perkin-Elmer Applied Biosystem) performed by ACCUGENIX® (Newark, DE)[9].

Growth Conditions and Isolation of the Compounds

The strain UMA-044 was cultivated in test tubes (i.d. 25×200 mm) containing 10 ml of medium (glucose 1.0%, peptone 0.5%, beef extract 0.5%, NaCl 0.3%, pH 7.0) on a rotary shaker (Model 3529; Lab-Line, Melrose, IL) held at 27°C with shaking at 240 rpm for 3 days. The tube cultures were used as a seed culture (1%) for 500-ml Erlenmeyer flasks (40 total) containing 150 ml of the same medium and incubated at 27°C for 7 days on a rotary shaker (220 rpm). The total cultured broth (6 liters) was filtered and the filtrate was extracted with ethyl acetate (EtOAc). The residue was extracted with methanol (MeOH) and evaporated *in vacuo* then extracted with EtOAc. The combined EtOAc extract (3.1 g) was subjected to repeated silica gel and reversed-phase column chromatography. Purification with guidance of cell-cell adhesion inhibition assay yielded three metabolites (**1**~**3**) as shown in Fig. 2. Among these compounds, bohemamine (**3**) is a pyrrolizidine alkaloid whose pharmacological effect has not reported previously[7].

NP 25301 (**1**): Physico-chemical properties, the ^{1}H-NMR and ^{13}C-NMR chemical shifts of **1** were shown in Tables 1 and 2, respectively.

Fig. 2. Fermentation of the strain UMA-044 and isolation of **1**~**3**.



NP25302 (**2**): Physico-chemical properties, the ^{1}H-NMR and ^{13}C-NMR chemical shifts of **2** were shown in Tables 1, 3 and 4, respectively.

Bohemamine (**3**): Colorless amorphous solid; m.p. 196~198°C ($CHCl_3$) (lit 199~200°C)[7]; Rf 0.36 (silica gel, $CHCl_3$-MeOH, 10 : 1), 0.57 (silica gel, EtOAc-Hexane,

8 : 1), 0.61 (reversed phase C_{18}, CH_3CN-H_2O, 3 : 7); HR-ESIMS calcd. for $C_{14}H_{18}O_3N_2$ $[M+H]^+$ 263.1396, found 263.1379. The ^{1}H-NMR and ^{13}C-NMR chemical shifts of **3** were shown in Tables 3 and 4, respectively.

Cell Culture and Antibodies

The cell lines used in this study were HL-60 (ATCC) and CHO-ICAM-1 (ATCC). 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, with or without retinoic acid (1 mM, Sigma).

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 a 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 (Santa Cruz Biotech, Inc., Santa Cruz, CA) 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[6]

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 an emission at 519 nm. Anti-LFA-1 and anti-ICAM-1 monoclonal antibodies were used as positive controls.

MTT Assay for Cytotoxicity of CHO-ICAM-1 Cells

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed using the method described by ALLEY *et al.*[15]. Briefly, 5×10^4 of CHO-ICAM-1 cells in 225 μl were plated into a 96-well microtiterplate. One day after incubation at 37°C under a 5% CO_2: 95% air atmosphere, 25 μl of each sample solution was added to each well and the plate was reincubated for 2 days. After cells were exposed to the test materials, 25 μl of MTT stock solution (5 mg/ml in PBS) was added to each well and the plate was incubated at 37°C for 4 hours. After aspiration of the medium, 100 μl of 0.04 N HCl-isopropanol was added to each well and mixed for 20 minutes at room temperature, the plate was read on a microplate reader using a test wavelength of 570 nm (reference wavelength at 630 nm).

Results

Strain Taxonomy

Based upon analysis of 16S RNA gene sequences analysis of the strain UMA-044, a phylogenetic tree was constructed, which visually illustrates the relatedness of the organ including 10 closest matches to the MicroSeq® data base (Fig. 3). Colonies of the strain UMA-044 are small (1~5 mm), lichenoid, and produce white to gray aerial mycelia and melanoid pigments. Though further studies based on the spore chain morphological properties using a scanning electron microscope, the type of diaminopimelic acid, the cultural and physiological characteristics and utilization of carbon sources were not performed, the strain UMA-044 was considered to belong to the genus *Streptomyces*.

Fig. 3. Phylogenic analysis of the strain UMA-044 based on 500 bp of 16S rRNA gene sequences.

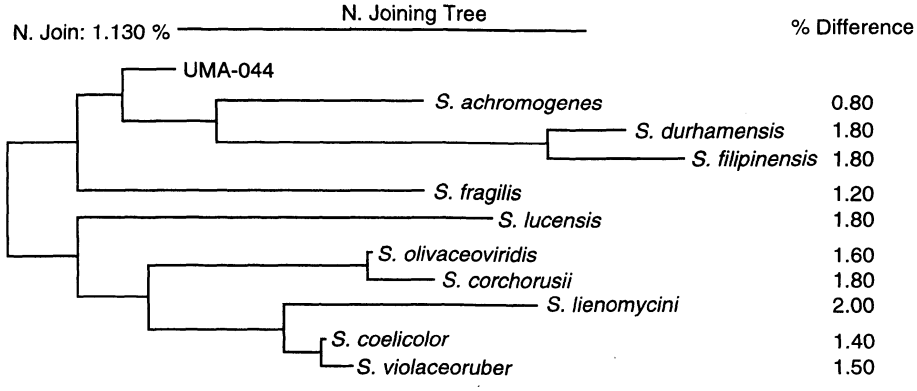


Table 1. Physico-chemical data of **1** and **2**.

	1	**2**
Appearance	Colorless amorphous solid	Colorless amorphous solid
M.p.	95-97 °C ($CHCl_3$)	229-230 °C ($CHCl_3$)
$[\alpha]_D^{24}$	–	+ 115.5° (*c* 1.1 MeOH)
Molecular formula	$C_{11}H_{11}NO_4$	$C_{14}H_{20}N_2O_2$
Molecular weight	221	248
HR-ESIMS *$(M+H)^+$	Found. 222.0759 ($C_{11}H_{11}NO_4$) Calcd. 222.0761	Found. 249.1602 ($C_{14}H_{21}N_2O_2$)* Calcd. 249.1603
UV λ_{max}^{MeOH} nm	284, 238, 261 (sh.)	334, 282, 250
IR ν_{max}^{KBr} cm^{-1}	3351, 3187, 1733, 1662, 1580, 1445, 1400 1325, 1235, 1156	3286, 3183, 2971, 2929, 1714, 1639, 1622, 1575, 1494, 1457, 1126
Color reaction		
50% H_2SO_4 + Δ	Positive	Positive
Iodine	Positive	Positive
Ehrlich's reagent + Δ	Positive	Positive
Dragendorff's reagent	Negative	Positive
TLC (Rf values)		
$CHCl_3$-MeOH[a] (10 : 1)	0.40	0.33
EtOAc-Hexane[a] (8 : 1)	0.31	0.40
CH_3CN-H_2O[b] (3 : 7)	0.32	0.16

[a] Silica gel TLC, [b] reversed phase TLC.

Structures of NP25301 (**1**) and NP25302 (**2**)

Physico-chemical properties of NP25301 (**1**) and NP25302 (**2**) were summarized in Table 1. NP25301 (**1**) was obtained as a colorless amorphous solid. The molecular formula of **1** was determined as $C_{11}H_{11}NO_4$ by HR-ESI mass spectrum. The IR spectrum of **1** showed absorptions due to a primary amide (3351, 3187, 1662 cm^{-1}), a conjugated ester carbonyl (1733 cm^{-1}), and aryl (1580, 1445 cm^{-1}) moieties. The ^{13}C-NMR displayed 11 carbons, indicating one methoxy, one exomethylene, four aromatic methine, and five quaternary carbons,

Table 2. ^{13}C- and ^{1}H-NMR data of NP25301 (**1**)[a].

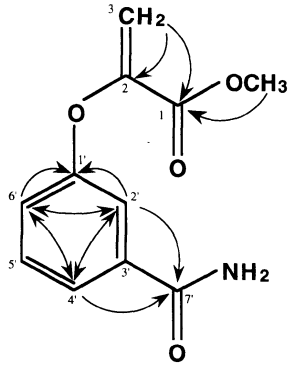
C		M	H (M, *J* value in Hz)
1	163.4	s	
2	150.1	s	
3	106.8	t	5.77 (1H, d, 2.1)
			4.99 (1H, d, 2.1)
1′	156.2	s	
2′	118.3	d	7.48 (1H, t, 1.8)
3′	135.8	s	
4′	123.4	d	7.54 (1H, dd, 7.7, 1.7)
5′	130.4	d	7.36 (1H, t, 7.9)
6′	122.6	d	7.13 (1H, dd, 8.1, 1.8)
7′	169.3	s	
1-OMe	53.0	q	3.78 (3H, s)
NH_2	–	–	6.58 (2H, br.s)

[a] δ ppm from TMS in $CDCl_3$; M, Multiplicity

assigned by DEPT experiment. In the ^{1}H-NMR spectrum of **1**, four signals for four aromatic protons at δ 7.48 (1H, t, J=1.8 Hz, C-2′), δ 7.54 (1H, dd, J=7.7, 1.7 Hz, C-4′), δ 7.36 (1H, t, J=7.9 Hz, C-5′), and δ 7.13 (1H, dd, J=8.1, 1.8 Hz, C-6′), were observed. The multiplicities of these aromatic protons were characteristic of a 1,3-disubstituted benzene moiety. The presence of an oxygenated aromatic carbon at C-1′ (δ 156.2, s) and a primary amide carbon at C-7′ (δ 169.3, s) were indicated from ^{13}C-NMR spectrum. The ^{1}H- and ^{13}C-NMR spectra of **1** displayed signals for an exomethylene (δ_C 106.8, t; δ_H 5.77 and 4.99, 1H, each, d, J=2.1 Hz), a carbomethoxy group (δ_C 53.0, s; δ_H 3.78, 3H, s) and two quaternary carbon atoms (δ 150.1 and δ 163.4), suggesting the presence of a methyl acrylate fragment[16]. The ^{1}H- and ^{13}C-NMR signals (Table 2) of **1**, a new compound identified as 2-(3′-carbamoylphenoxy)acrylic acid methyl ester, was unambiguously assigned using HMQC and HMBC NMR correlations (Fig. 4).

NP 25302 (**2**) was isolated as a colorless amorphous solid. The molecular formula of **2** was determined as $C_{14}H_{20}N_2O_2$ by HR-ESI-MS. The UV spectrum of **2** exhibited three maxima at 334, 282, and 250 nm, which were similar to those of the known compound bohemamine (**3**) (lit. 335, 286, 248 nm)[7] isolated together with other compounds. The IR bands at 3286, 3183, 1714, 1639, 1575 and 1494 cm^{-1} were also characteristic of bohemamine (**3**) with an α,β-unsaturated secondary amide and a five membered α,β-unsaturated ketone[7]. Comparison of the NMR data of **2** with that of **3** (Tables 3 and 4) indicated that they are related. The signals of C-5 and C-6 in **3** (δ_H 3.58, 2H, d, J=3.4 Hz and δ_C 56.7 and 56.4, d each) were replaced in **2** by ^{1}H-NMR signals at δ 1.83 (1H, m, H-5α) and δ 2.46 (1H, m, H-5β), and δ 1.85 (1H, m, H-6α) and δ 1.69 (1H, m, H-6β) and by ^{13}C-NMR signals at δ 35.8 (t, C-5) and d 28.7 (t, C-6), indicating two methylene groups. From these results, compound **2** was identified to be the deoxy derivative of **3**. Comparison of the molecular weight of **2** (*m/z* 248) with that of **3** (*m/z* 262) also suggested loss of an oxygen atom corresponding to an epoxy moiety (*m/z* 14 units) in **3**. Final elucidation of **2** was performed using HMBC correlation (Fig. 5). The relative stereochemistry of protons attached to carbons at C-4, 5, 6 and 7 was established based on comparison of NOESY experiment of **2** with that of **3**. In the NOESY spectrum of **2** (Fig. 6), the correlation between a methyl signal at C-7 and a proton signal at H-4β were recognized through a proton signal at H-5β. Therefore, the methyl group attached to C-4 carbon in **2** should have an α configuration same as that of bohemamine (**3**). Consequently, the structure of NP25302 (**2**) was determined as deoxybohemamine (Fig. 1).

Fig. 4. ^{1}H-^{13}C long range coupling detected by HMBC experiment of **1**.



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

The inhibitory effect of NP25301 (**1**) NP25302 (**2**) and bohemamine (**3**) on cell aggregation, cell adhesion, and cell proliferation are shown in Table 5. Compound **1** was a potent inhibitor on cell aggregation, while compounds **1**, **2** and **3** inhibited adhesion of HL-60 cells to CHO-ICAM-1

Table 3. ^{13}C- and ^{1}H-NMR data of NP25302 (**2**) and bohemamine (**3**)[a].

C	2	M	3	M	Δ (2 - 3)
1	205.7	s	202.1	s	3.6
2	94.0	d	93.1	d	0.9
3	167.7	s	170.0	s	- 2.3
4	55.2	d	64.2	d	-9.0
5	35.8	t	56.7	d	-20.9
6	28.7	t	56.4	d	-27.7
7	75.1	s	74.6	s	0.5
1′	164.6	s	162.7	s	1.9
2′	118.1	d	117.8	d	0.3
3′	158.6	s	159.8	s	-1.2
4′	28.1	q	28.2	q	-0.1
5′	20.9	q	21.0	q	-0.1
4-*Me*	17.8	q	14.8	q	3.0
7-*Me*	25.0	q	19.6	q	5.4

[a] δ ppm from TMS in $CDCl_3$; M, Multiplicity

Table 4. ^{1}H-NMR data of NP25302 (**2**) and bohemamine (**3**)[a].

H	2 (M, *J* value in Hz)	3 (M, *J* value in Hz)
2	5.75 (1H, s)	5.69 (1H, s)
4	4.08 (1H, m)	3.94 (1H, q, 6.1)
5α	1.83 (1H, m)	–
5β	2.46 (1H, m)	3.58 (2H, d, 3.4)
6 α	1.85 (1H, m)	–
6 β	1.69 (1H, m)	3.58 (2H, d, 3.4)
2′	6.01 (1H, s)	5.98 (1H, s)
4′	1.93 (3H, s)	2.20 (3H, s)
5′	2.23 (3H, s)	1.91 (3H, s)
4-*Me*	1.18 (3H, d, 6.5)	1.58 (3H, d, 6.5)
7-*Me*	1.34 (3H, s)	1.37 (3H, s)
–N*H*–	9.91 (1H, br.s)	9.57 (1H, br.s)

[a] δ ppm from TMS in $CDCl_3$; M, Multiplicity

cells at a concentration range of 24~30 μg/ml without cytotoxicity, at least up to 50 μg/ml.

Discussion

In this study, we used a combination of two different methods to screen inhibitors of cell adhesion between LFA-1 and ICAM-1 as reported in a previous paper[6]. The primary method used was the cell aggregation inhibitory assay based on HL-60 cells, 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 cells and ICAM-1 of CHO-ICAM-1 cells. In the course of our screening program searching for inhibitors of cell adhesion molecule from microbial organisms, we examined a cultured broth of *Streptomyces* sp. UMA-044. Two new compounds NP25301 (**1**) and NP25302 (**2**) were isolated and their structures were determined to be 2-(3′-carbamoylphenoxy)acrylic acid methyl ester for **1** and deoxybohemamine for **2**. NP25301 (**1**) has a unique

structure consisting of a benzamide and an acrylic acid methyl ester. As far as we know, 3-chloro-4-hydroxy-5-farnesyl-benzamide isolated from an unidentified South African soil fungus, was the only reported microbial metabolite possessing a benzamide moiety[17]. The known compound bohemamine (**3**) previously isolated from *Actinosporangium* sp. strain C36145 (ATCC 31127), was reported to be inactive against bacteria, fungi and tumours[7]. This is the first report of cell adhesion inhibitory activity of bohemamine derivatives.

Compounds **1**~**3** showed inhibition of PMA-induced cell aggregation of HL-60 cells, without cytotoxicity at least up to 50 μg/ml. Furthermore, compounds **1**~**3** inhibited adhesion of HL-60 cells to CHO-ICAM-1 cells at IC_{50} values of 29.5 for **1**, 24.3 for **2** and 27.2 μg/ml for **3**. The positive control, cytochalasin B, showed potent inhibitory activity at IC_{50} values of 6.4 μg/ml. However, cytotoxic effect of cytochalasin B on CHO-ICAM-1 cells was shown in 24 hours exposure at IC_{50} value of 1.8 μg/ml. On the

Fig. 5. Key ^{1}H-^{13}C long range couplings detected by HMBC experiment and assignment of quarternary carbon atoms at C-1, C-3, C-1′ and C-3′ of **2**.

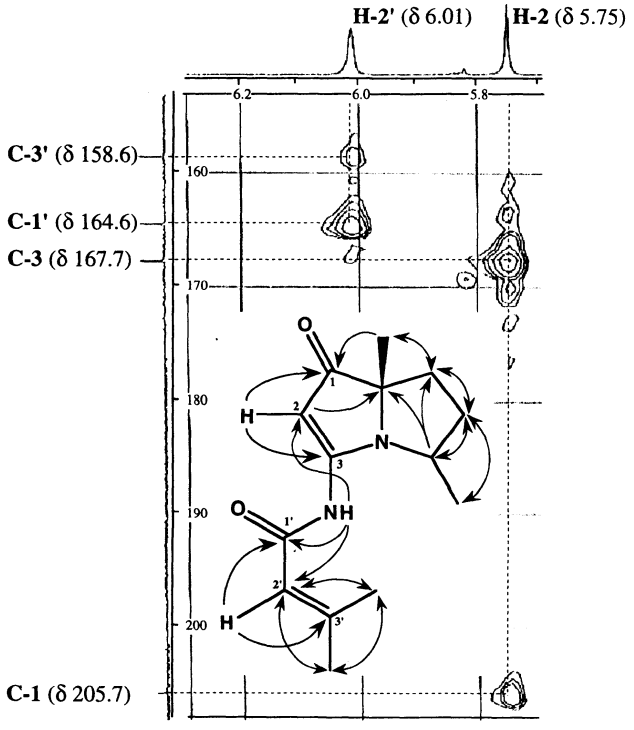


Fig. 6. NOESY correlation for **2**.

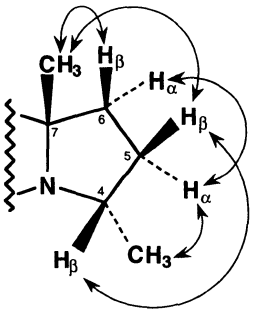


Table 5. Effect of **1**~**3** on cell aggregation, cell proliferation, and cell adhesion.

	HL-60			CHO-ICAM-1		
	Cell Aggregation (A)	Cell Proliferation (B)*		Cell Adhesion (C)	Cell Proliferation (D)**	
	MIC (μg/ml)	IC_{50} (μg/ml)	S.I. (B)/(A)	IC_{50} (μg/ml)	IC_{50} (μg/ml)	S.I. (D)/(C)
1.	41.7	95.0	2.3	29.5	78.0	2.6
2.	125	>125	>1.0	24.3	75.0	3.1
3.	125	> 125	>1.0	27.2	60.0	2.2
Cyt. B	6.9	> 62.5	>9.1	6.4	1.8	0.3
Dexa	–	> 62.5	–	–	50.0	–

MIC, Minimum Inhibitory Concentration; *XTT and **MTT assay; S.I., Specific Index; –, inactive, Cyt B, cytochalasin B; Dexa, Dexamethasone.

other hand, compound **1**~**3** did not show significant cytotoxic effect on CHO-ICAM-1 cells at least up to 50 μg/ml. It is of interest to consider a structure-activity relationship (SAR) between a nitrogen atom containing skeleton and cell adhesion activity on NP25301 (**1**) and NP25302 (**2**) as well as a β-carboline alkaloid, pyridindolol K2 from *Streptomyces* sp. K93-0711[18].

Most recently, *N*-bromobenzoyl L-tryptophan was identified as an inhibitor to the formation of the LFA-1/ICAM complex, using transfer technology of essential protein epitopes of ICAM-1 for interaction with LFA-1 to a small molecule[19,20]. The SAR of amino acid moiety in a molecule was reported in particular. NP25301 (**1**) and 2-bromobenzoyl moiety of *N*-bromobenzoyl L-tryptophan are partial strucurally-related. Therefore, it is matter of deep interest that the SAR of 2-bromobenzoyl moiety will be clarified.

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