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Cell-adhesion Inhibitors Produced by a Sea Hare-derived *Periconia* sp.

III Absolute Stereostructures of Peribysin J and Macrosphelide M

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Abstract Peribysin J and macrosphelide M have been isolated from a strain of *Periconia byssoides* originally isolated from the sea hare *Aplysia kurodai*. Their absolute stereostructures have been elucidated on the basis of spectroscopic analyses using 1D and 2D NMR techniques and some chemical transformations including the modified Mosher's method. These fungal metabolites inhibited the adhesion of human-leukemia HL-60 cells to human-umbilical-vein endothelial cells (HUVEC).

Keywords fungus, cell-adhesion inhibitor, peribysin, macrosphelide, *Periconia* sp.

Introduction

Based on the fact that some of the bioactive materials isolated from marine animals have been produced by bacteria, we have turned our attention to new antitumour materials from microorganisms inhabiting the marine environment [1~3]. As part of this study, we have previously isolated the cell-adhesion inhibitors, macrosphelides E~I, L [4, 5] and peribysins A~I [6~8], from a strain of *Periconia byssoides* OUPS-N133 originally isolated from the sea hare *Aplysia kurodai*. All of these compounds, except for macrosphelide I, inhibited the adhesion of human-leukemia HL-60 cells to human-umbilical-vein endothelial cells (HUVEC) more potently than herbimycin A [9, 10]. Further investigation of the metabolites from this fungal strain has now led to the isolation of new anti-adhesion compounds designated peribysin J (**1**) and macrosphelide M (**2**) (Fig. 1). We describe herein the absolute stereostructures of **1** and **2** (Fig. 1) in addition to their inhibition of cell adhesion.

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Results and Discussion

The fungal strain was cultured at 27°C for 4 weeks in a medium containing malt extract 1.0%, glucose 1.0% and peptone 0.05% in artificial seawater adjusted to pH 7.5, as reported previously [4~8]. The AcOEt extract of the culture filtrate was purified by chromatography on Sephadex LH-20 and silica gel by reversed phase HPLC to afford **1** and **2**. The physico-chemical properties of these compounds are summarized in Table 1.

1 had the molecular formula $C_{15}H_{26}O_5$ established by the $[M]^+$ peak of **1** in high-resolution electron-impact mass spectrometry (HREI-MS). Its IR spectrum exhibited bands at 3384 and 1653 cm^{-1}, characteristic of an alcohol and a double bond. A close inspection of the ^1H- and ^{13}C-NMR

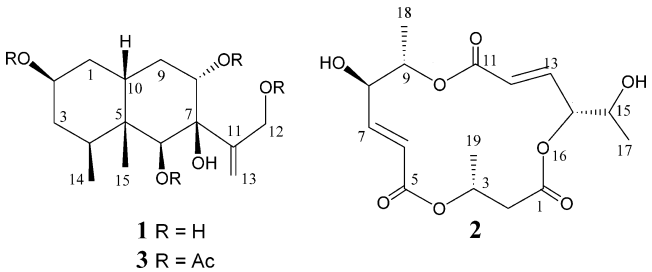


Fig. 1 Structures of peribysin J (**1**) and macrosphelide M (**2**).

Table 1 Physico-chemical properties of peribysin J (**1**) and macrosphelide M (**2**)

	1	**2**
Appearance	Pale yellow oil	Pale yellow oil
$[\alpha]_D^{22}$	−190.1 (*c* 0.28, EtOH)	+5.5 (*c* 0.30, EtOH)
HREI-MS		
Found:	286.1775 $(M)^+$	343.1395 $(M+H)^+$
Calcd:	286.1773 (for $C_{15}H_{26}O_5$)	343.1392 (for $C_{16}H_{23}O_8$)
Molecular formula	$C_{15}H_{26}O_5$	$C_{16}H_{22}O_8$
UV λ_{max}^{EtOH} nm (log ε)	208 (3.38)	212 (4.09)
IR ν_{max} (neat) cm^{-1}	3384, 1653	3450, 1722, 1717, 1658, 1652
TLC Rf [a]	0.12	0.48
Solubility		
soluble	DMSO, MeOH, Me_2CO	DMSO, MeOH, Me_2CO
insoluble	CH_2Cl_2, H_2O	H_2O

[a] Silica gel (10% MeOH in CH_2Cl_2).

Table 2 NMR spectral data of peribysin J (**1**) in MeOH-d_4

Position	δ_H [a]	*J*/Hz	^1H-^1H COSY	NOE	δ_C	HMBC (C) [b]
1α	1.68 dt	12.4 (1β), 4.8 (2, 10)	1β, 2, 10	1β, 2, 10	37.38 (t)	2, 5
β	1.58 td	12.4 (1α, 2), 4.8 (10)	1β, 2, 10	1α, 10,15		2
2	3.74 tdd	12.4 (1β, 3β), 4.8 (1α), 2.7 (3α)	1α, 1β, 3α, 3β	1α, 3α, 4, 9α	67.19 (d)	
3α	1.60 ddd	12.4 (3β), 3.9 (4), 2.7 (2)	2, 3β	2, 4, 3β, 14	40.97 (t)	2
β	1.22 td	12.4 (2, 3α), 11.0 (4)	2, 3α, 4	3α, 14, 15		2, 4, 5, 14
4	2.07 dqd	11.0 (3β), 6.8 (14), 3.9 (3α)	3β, 14	2, 3α, 6, 9α, 14	33.24 (d)	3, 14, 15
5					42.25 (s)	
6	3.80 s			4, 12A, 12B, 13B, 14	77.27 (d)	4, 8, 10, 15
7					80.42 (s)	
8	3.87 dd	10.9 (9α), 5.4 (9β)	9α, 9β	9β, 10	75.81 (d)	7, 11
9α	2.01 td	12.8 (9β, 10), 10.9 (8)	8, 9β, 10	2, 4, 9β, 13B	35.42 (t)	8, 10
β	1.71 ddd	12.8 (9α), 5.4 (8), 4.8 (10)	8, 9α, 10	8, 9α, 10		1, 7
10	1.58 dq	12.8 (9a), 4.8 (1α, 1β, 9β)	1α, 1β, 9α, 9β	1α, 1β, 8, 9β, 15	38.77 (d)	
11					150.30 (s)	
12A	4.19 d	13.6 (12B)	12B	6, 12B, 13A	64.46 (t)	11, 13
B	4.32 d	13.6 (12A)	12A	6, 12A, 13A		11, 13
13A	5.46 s			12A, 12B, 13B	117.19 (t)	7, 12
B	5.66 s			9α, 6, 13A		7, 12
14	0.85 d	6.8 (4)	4	3α, 3β, 4, 6, 15	17.51 (q)	3, 4, 5
15	1.00 s			1α, 3β, 10, 14	17.51 (q)	4, 5, 6, 10

[a] ^1H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constants (*J*/Hz). Figures in parentheses indicate the proton coupling with that position. [b] Long range ^1H-^{13}C correlations from H to C observed in the HMBC experiment.

spectra of **1** (Table 2) by DEPT and HMQC experiments revealed the presence of one vinylidene (C-11 and C-13), one secondary methyl (C-14), one tertiary methyl (C-15), four sp^3-hybridized methylenes (C-1, C-3, C-9 and C-12) including one oxymethylene (C-12), five sp^3-methines (C-2, C-4, C-6, C-8 and C-10) including three oxymethines (C-2, C-6 and C-8), two quarternary sp^3-carbons (C-5 and C-7) including one oxygen-bearing carbon (C-7). The ^1H-^1H COSY analysis of **1** led to a partial structural unit as shown by bold-faced lines in Fig. 2, and supported by HMBC

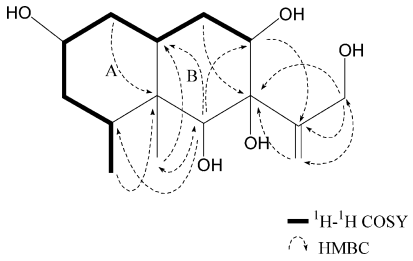


Fig. 2 Selected ^{1}H-^{1}H COSY and HMBC correlations in peribysin J (**1**).

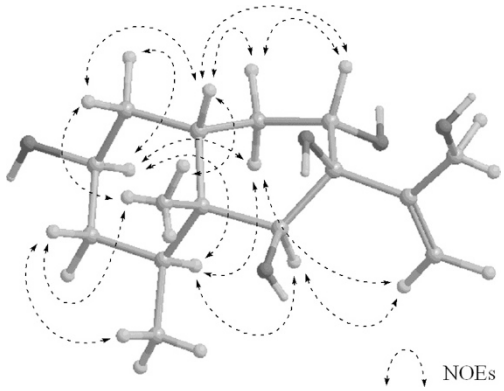


Fig. 3 Observed NOEs for peribysin J (**1**).

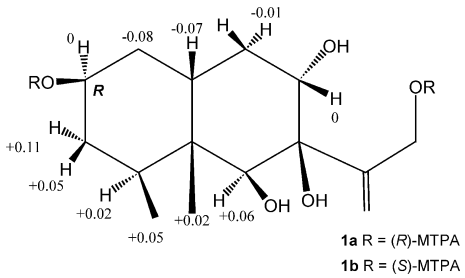


Fig. 4 ^{1}H chemical-shift differences ($\Delta\delta=\delta_S-\delta_R$) between the (*R*)- and (*S*)-MTPA esters (**1a** and **1b**) of peribysin J (**1**).

correlations (Table 2). The connection of these units and the remaining functional groups was determined on the basis of the key HMBC correlations summarized in Fig. 2.

The acetylation of **1** gave tetraacetate **3**, and the proton signals for 2-H, 6-H, 8-H and H-12 were downfield-shifted from δ_H 3.74, δ_H 3.80, δ_H 3.87 and δ_H 4.19, 4.32 to δ_H 4.92, δ_H 5.66, δ_H 5.18 and δ_H 4.74, respectively. This observation, together with the molecular formula of **1**, implied that the positions of the hydroxyl groups were at C-2, C-6, C-8 and C-7 (δ_C 80.42), thus elucidating the planar structure of **1**.

The relative stereochemistry of **1** was deduced from NOESY experiments (Fig. 3). NOE correlations from 15-H to 1β-H and 3β-H implied the A ring to exist in a chair conformation with the 5-methyl group, 1β-H and 3β-H in coaxial arrangements. NOEs correlations (15-H/10-H, 14-H/3β-H and 4-H/2-H) suggested the 5-methyl group to be oriented *cis* to 10-H, and the 2-hydroxyl group and the 4-methyl group to be arranged equatorially. NOEs from 6-H to 4-H and from 9α-H to 4-H and from 8-H to 10-H implied that the B ring exists in a twist-boat conformation, and that 6-H is oriented *cis* to both the C-4–C-5 bond and the 8-hydroxyl group. In addition, NOEs correlations (6-H/13A-H and 9α-H/13A-H) suggested that the C-7–C-11 bond is oriented *cis* to 6-H. Consequently, the relative stereostructure for **1** can be depicted as shown in Fig. 3.

The modified Mosher method [11] was applied to determine the absolute configuration of **1**. The ^{1}H chemical-shift differences between the (*R*)- and (*S*)-MTPA esters (**1a** and **1b**) of **1** are shown in Fig. 4. The result suggested that **1** has the 2*R* configuration. Hence the absolute stereostructure of **1** was determined as 2*R*, 4*S*, 5*R*, 6*S*, 7*R*, 8*S*, 10*R*.

2 was assigned the molecular formula $C_{16}H_{22}O_8$ deduced from HREI-MS. Its IR spectrum exhibited bands at 3450, 1722, 1717, 1658 and 1652 cm^{-1}, characteristic of hydroxy and carbonyl groups, and a double bond. A close inspection of the ^{1}H- and ^{13}C-NMR spectra of **2** (Table 3) by DEPT and HMQC experiments revealed the presence of three secondary methyls (C-17, C-18 and C-19), one sp^3-hybridized methylene (C-2), five oxygen-bearing sp^3-methines (C-3, C-8, C-9, C-14 and C-15), two 1,2-disubstituted double bonds (C-6, C-7, C-12 and C-13) and three ester carbonyl groups. The ^{1}H-^{1}H COSY analysis of **2** led to three carboxylic acid units as shown by bold faced lines in Fig. 5, which was supported by HMBC correlations (Table 3). The *E*-geometry of both the Δ^6- and Δ^{12}-double bonds was deduced from the coupling constants ($J_{6,7}$ 15.8 Hz and $J_{12,13}$ 15.2 Hz) of the olefinic protons. The connection of these three units and remaining ester moiety was determined on the basis of the key HMBC correlations (7-H/C-5, 6-H/C-5, 3-H/C-5, 3-H/C1, 2-H/C-1, 14-H/C-1, 13-H/C-11, 12-H/C-11 and 9-H/C-11) (Table 3, Fig. 5), leading to the planar structure of **2**.

Since the stereostructure of **2** could not be deduced from NOESY experiments, **2** was degraded to the methyl esters of the constituent carboxylic acids. Acid-catalyzed methanolysis of **2** in MeOH gave only two products, methyl esters of 3-hydroxybutyric acid and 4,5-dihydroxyhex-2*E*-enoic acid, which were isolated as *p*-bromobenzoates (respectively **4** and **5**) because of the volatility of methyl 3-

Table 3 NMR spectral data of macrosphelide M (**2**) in Me_2CO-d_6

Position	δ_H[a]	*J*/Hz	^{1}H-^{1}H COSY	δ_C	HMBC (C)[b]
1				169.08 (s)	
2A	2.51 dd	15.2 (2B), 10.6 (3)	2B, 3	42.12 (t)	1, 3, 19
B	2.89 dd	15.2 (2A), 4.1 (3)	2A, 3		1
3	5.28 dqd	10.6 (2A), 6.0 (19), 4.1 (2B)	2A, 2B, 19	68.99 (d)	1, 5
5				166.22 (s)	
6	6.08 dd	15.8 (7), 2.2 (8)	7	122.92 (d)	5, 8
7	7.04 dd	15.8 (6), 4.5 (8)	6, 8	147.26 (d)	5
8	4.47 br s		7, 9, 8-OH	75.11 (d)	6, 7, 9, 18
9	5.12 qd	6.2 (18), 3.0 (8)	8, 18	75.24 (d)	8, 11
11				164.71 (s)	
12	5.89 dd	15.2 (13), 1.1 (14)	13	126.16 (d)	11, 14
13	6.47 dd	15.2 (12), 8.8 (14)	12, 14	144.39 (d)	11
14	5.18 ddd	8.8 (13), 5.5 (15), 1.1 (12)	13, 15	78.19 (d)	1, 12
15	3.90 qdd	6.0 (17), 5.5 (14), 4.9 (15-OH)	14, 17, 15-OH	68.49 (d)	
17	1.14 d	6.0 (15)	15	19.12 (q)	14, 15
18	1.39 d	6.2 (9)	9	17.95 (q)	8, 9
19	1.36 d	6.0 (3)	3	20.08 (q)	2, 3
8-OH	4.56 d	4.9 (8)	8		7, 9
15-OH	4.17 d	4.9 (15)	15		17

[a] and [b] as in Table 1.

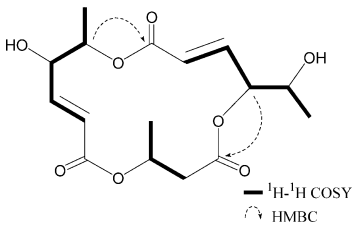


Fig. 5 Selected ^{1}H-^{1}H COSY and HMBC correlations in macrosphelide M (**2**).

hydroxybutyrate (Scheme 1). This fact demonstrated that the two dihydroxycarboxylic acid moieties of **2** have the same stereochemistry. These degradation products from **2** were identical with those derived from macrosphelide E by comparison of spectral data reported previously (Scheme 1) [4]. Hence, **4** and **5** were identified as methyl (3*R*)-3-(*p*-bromobenzoyloxy)butyrate and methyl (4*R*,5*S*)-4,5-di(*p*-bromobenzoyloxy)hex-2*E*-enoate, respectively. The above summarized evidence led to the absolute stereostructure **2** for macrosphelide M.

1 and **2** were evaluated in the adhesion assay system using HL-60 cells and HUVEC, according to a modification of the method reported by Miki and co-workers [12]. As shown in Table 4, these compounds inhibited the adhesion of HL-60 cell to HUVEC more potently than herbimycin A [9, 10].

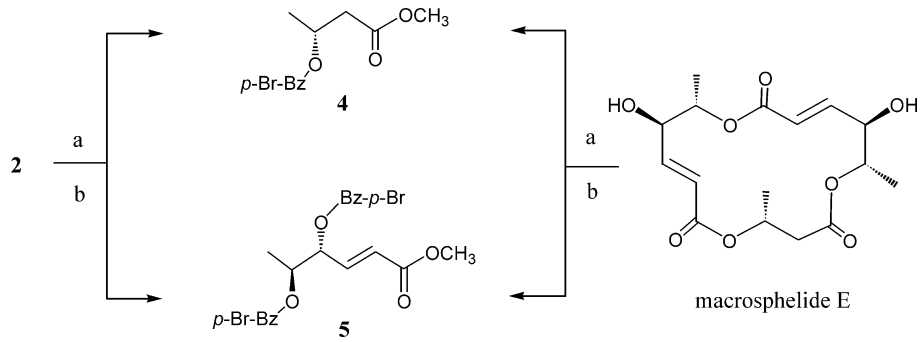
Experimental

General

UV spectra were recorded on a Shimadzu spectrophotometer and IR spectra on a Perkin Elmer FT-IR spectrometer 1720X. NMR spectra were recorded at 27°C on Varian UNITY INOVA-500 and MERCURY spectrometers with tetramethylsilane (TMS) as an internal reference. EI-MS was determined using a Hitachi M-4000H mass spectrometer. Optical rotations were recorded on a JASCO J-820 polarimeter. Liquid chromatography over silica gel (mesh 230~400) was performed at medium pressure. HPLC was run on a Waters ALC-200 instrument equipped with a differential refractometer (R 401) and Shim-pack PREP-ODS (25 cm×20 mm i.d.). Analytical TLC was performed on precoated Merck aluminium sheets (DC-Alufolien Kieselgel 60 F254, 0.2 mm) with the solvent system CH_2Cl_2 - MeOH (9 : 1), and compounds were viewed under UV lamp and sprayed with 10% H_2SO_4 followed by heating.

Culturing and Isolation of Metabolites

A strain of *P. byssoides* OUPS-N133, isolated from the sea



Scheme 1 Reaction conditions: (a) MeOH, H_2SO_4; (b) *p*-Br-BzCl, pyridine.

Table 4 Inhibitory activity of cell adhesion of metabolites

Compound	IC_{50} (μM)
Peribysin J (**1**)	11.8
Macrosphelide M (**2**)	33.2
Herbimycin A (control)	38.0

hare *A. kurodai*, was cultured at 27°C for four weeks in a liquid medium (90 liters) containing malt extract 1.0%, glucose 1.0% and peptone 0.05% in artificial seawater adjusted to pH 7.5. As reported previously [4], the EtOAc extract (5.7 g) of the culture filtrate was successively chromatographed on Sephadex LH-20 (CH_2Cl_2 - MeOH, 1 : 1) and silica gel (CH_2Cl_2 - MeOH). The MeOH - CH_2Cl_2 (10 : 90) eluate (254.6 mg) from silica gel column chromatography was purified by HPLC using MeOH - H_2O (40 : 60) as the eluent to afford **1** (8.8 mg, 0.15%), and the MeOH - CH_2Cl_2 (5 : 95) eluate (315.7 mg) from silica gel column chromatography was purified by HPLC using MeOH - H_2O (50 : 50) as the eluent to afford **2** (11.1 mg, 0.19%).

Acetylation of 1

To a solution of **1** (2.8 mg) in pyridine (1.0 ml) was added Ac_2O (1.0 ml), and the reaction mixture was left at room temperature overnight. The mixture was evaporated under reduced pressure and residue was purified by HPLC using MeOH - H_2O (70 : 30) as the eluent to afford tetraacetate **3** (1.8 mg) as a pale yellow oil.

Tetraacetate **3**: EI-MS *m/z* 454 (M^+, 2.5%); HREI-MS *m/z* for $C_{23}H_{34}O_9$ (M^+), Calcd: 454.2193; Found: 454.2196; ^{1}H-NMR δ ppm ($CDCl_3$): 0.87 (3H, d, J=6.6 Hz, 14-H), 0.97 (3H, s, 15-H), 1.36 (1H, q, J=11.8 Hz, 3β-H), 1.64 (2H, m, 1-H), 1.81 (1H, dt, J=11.8, 4.8 Hz, 3α-H), 1.82 (1H, m, 10-H), 2.01 (3H, s, COOCH_3), 2.06 (3H, s, COOCH_3), 2.06 (2H, m, 9-H), 2.10 (1H, dqd, J=11.8, 6.6, 4.8 Hz, 4-H), 2.11 (6H, s, COOCH_3), 2.11 (1H, br s, 2-OH), 4.74 (2H, s, 12-H), 4.92 (1H, tt, J=11.8, 4.8 Hz, 2-H), 5.18 (1H, dd, J=10.3, 5.1 Hz, 8-H), 5.43 (1H, s, 13A-H), 5.46 (1H, s, 13B-H), 5.66 (1H, br s, 6-H).

Formation of the (*R*)- and (*S*)-MTPA Esters 1a and 1b from 1

(*R*)-MTPA (2.0 mg), dicyclohexylcarbodiimide (DCC) (2.0 mg) and 4-(dimethylamino)-pyridine (DMAP) (1.0 mg) were added to a CH_2Cl_2 solution (0.2 ml) of **1** (1.2 mg), and the reaction mixture was left at room temperature for 3 hours. The solvent was evaporated off under reduced pressure, and the residue was purified by HPLC using MeOH - H_2O (65 : 35) as the eluent to afford (*R*)-MTPA ester **1a** (0.9 mg) as an amorphous powder. The same reaction with **1** (1.4 mg) using (*S*)-MTPA (2.1 mg) gave ester **1b** (1.0 mg).

Ester **1a**: EI-MS *m/z* 700 (M^+, 1.2%); HREI-MS *m/z* for $C_{35}H_{40}F_6O_9$ (M^++H_2O), Calcd: 718.2565; Found: 718.2572; ^{1}H-NMR δ ppm ($CDCl_3$): 0.77 (3H, d, J=6.9 Hz, 14-H), 0.99 (3H, s, 15-H), 1.35 (1H, q, J=12.2 Hz, 3β-H), 1.71 (1H, m, 3α-H), 1.74 (2H, m, 1-H), 1.90 (1H, m, 9β-H), 1.98 (1H, m, 9α-H), 2.00 (1H, m, 4-H), 2.14 (1H, m, 10-H), 3.55 (6H, s, OCH_3), 3.80 (1H, s, 6-H), 4.00 (1H, dd, J=10.1, 5.6 Hz, 8-H), 4.90 (1H, d, J=13.8 Hz, 12A-H), 5.05 (1H, d, J=13.8 Hz, 12B-H), 5.18 (1H, m, 2-H), 5.47 (1H, s, 13A-H), 5.77 (1H, s, 13B-H), 7.41 (6H, m, Ar.H) and 7.52 (4H, m, Ar.H).

Ester **1b**: EI-MS *m/z* 700 (M^+, 0.9%); HREI-MS *m/z* for $C_{35}H_{40}F_6O_8$ (M^++H_2O), Calcd: 718.2565; Found: 718.2570; ^{1}H-NMR δ ppm ($CDCl_3$): 0.82 (3H, d, J=6.9 Hz, 14-H), 1.01 (3H, s, 15-H), 1.46 (1H, q, J=12.2 Hz, 3β-H), 1.66 (2H, m, 1-H), 1.76 (1H, m, 3α-H), 1.89 (1H, m, 9β-H), 1.90 (1H, m, 9α-H), 2.02 (1H, m, 4-H), 2.07 (1H, m, 10-H), 3.54 (3H, s, OCH_3), 3.56 (3H, s, OCH_3), 3.86 (1H, s, 6-H), 4.00 (1H, dd, J=10.1, 5.6 Hz, 8-H), 4.88 (1H, d, J=13.8 Hz, 12A-H), 5.03 (1H, d, J=13.8 Hz, 12B-H), 5.18 (1H, m, 2-H), 5.40 (1H, s, 13A-H), 5.74 (1H, s, 13B-H),

7.41 (6H, m, Ar.H) and 7.52 (4H, m, Ar.H).

***p*-Bromobenzoate 4 and 5 of Degradation Products from 2**

To a solution of **2** (2.6 mg) in MeOH (0.1 ml) was added conc. H_2SO_4 (0.02 ml), and the reaction mixture was left at room temperature overnight. The mixture was diluted with water and extracted with diethyl ether, and the extract was evaporated under reduced pressure to give a mixture of crude methyl carboxylate. To a solution of the mixture in pyridine (0.2 ml) was added *p*-bromobenzoyl chloride (3.2 mg), and the reaction mixture was left at room temperature overnight. The mixture was concentrated to dryness under reduced pressure, and the residue was purified by HPLC using MeOH - H_2O (90 : 10) as the eluent to afford *p*-bromobenzoates **4** (0.5 mg) and **5** (1.1 mg) as a pale yellow oil. The spectral data, including CD spectra, of **4** and **5** were identical with those of the degradation products from macrosphelide E reported previously [4].

Cell Adhesion Assay

This assay was carried out according to a modification of the Miki's method using 3-(4,5-di-methyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT)-labeled cells [12]. HUVEC (DIA-IATRON Co., Ltd.) were cultured until confluent in a 96-well plate in medium 199 (Gibco) containing 10% fetal calf serum (FCS, Gibco) and washed with phosphate buffered saline (PBS, DIA-IATRON Co., Ltd.) containing 20% FCS. The HUVEC were stimulated with a solution of lipopolysaccharides (LPS, Sigma) in RPMI 1640 medium (Gibco) containing 10% FCS for 4 hours in the presence of various concentrations of macrosphelides, and then MTT-labeled HL-60 cells were added and incuvated for 40 minutes at 37°C in 5.0% CO_2. Unbound cells were gently washed out with PBS containing 10% FCS, and DMSO was added to lyse the adherent HL-60 cells. Absorbance at 540 nm was measured using a microplate reader (Model 450, BIO-RAD).

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