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Fungal Metabolites, Asterric Acid Derivatives Inhibit Vascular Endothelial Growth Factor (VEGF)-induced Tube Formation of HUVECs

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In the search for new naturally occurring anti-angiogenic compounds, we found that a culture broth of an unidentified fungal strain B90911 exerted inhibitory activity on capillary-like tube formation of human umbilical vein endothelial cells (HUVEC) *in vitro*. Four active compounds were isolated by bioassay-guided separation and their structures were identified to be sulochrin (**1**), methyl asterric acid (**2**), and two new asterric acid derivatives, 3-chloroasterric acid (**3**), and 3,5-dichloroasterric acid (**4**) by spectroscopic analyses. These compounds significantly inhibited the VEGF-induced tube formation of HUVEC, suggesting that asterric derivatives could be useful for further study as anti-angiogenic agents.

Angiogenesis is found in physiological conditions including embryonic development, wound healing, and tissue regeneration. It is also associated with pathological diseases such as rheumatoid arthritis, intra-ocular vascular syndromes, diabetic retinopathy, psoriasis, and others[1]. Many experimental evidences support that tumor angiogenesis is fundamental for the growth and metastasis of solid tumors[2]. Therefore, inhibition of new vessel formation namely angiogenesis may lead to therapeutic regulation of tumor growth. *In vitro* angiogenesis assays are important for identification of potential angiogenic inhibitors and rapid screening for pharmacological inhibitors. In particular, differentiation of endothelial cells to capillary-like structure on Matrigel is known to mimic *in vivo* angiogenic processes. When endothelial cells are plated on Matrigel (a reconstituted basement membrane protein derived from the Engelbreth-Holm-Swarm mouse tumor), the cells stop proliferating, align, and form tubes proposed as models of endothelial cell differentiation, the final step of angiogenesis. These tubes are morphologically similar to capillaries. Thus, this *in vitro* angiogenesis assay is a very useful method to evaluate antiangiogenic activity of various compounds.

In our search for new anti-angiogenic microbial metabolites, a culture broth of an unidentified fungal isolate, B90911, exerted inhibitory effect on the tube formation of HUVECs. Bioassay-guided isolation led to identify four active principles namely sulochrin (**1**) and methyl asterric acid (**2**), known fungal metabolites and 3-chloroasterric acid (**3**) and 3,5-dichloroasterric acid (**4**), new compounds. Here we described the isolation, structure determination of compounds **1**~**4**, and their inhibitory activity of VEGF-induced tube formation of HUVECs.

Materials and Methods

Instruments and Chemicals

Melting points were measured on a Electrothermal model 9100 and are uncorrected. UV spectra were obtained on a Milton Roy 3000 spectrometer. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75.5 MHz) spectra were obtained on a Varian Unity NMR Spectrometer and chemical shifts were referenced to tetramethylsilane (TMS) as the internal standard. HMBC spectra were determined on a Brucker AMX 500 NMR spectrometer. ESI-MS was measured on a JMS-HS 110A (JEOL) mass spectrometer. HR-FAB-MS was measured on a JMS 110A/110A (JEOL) mass

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spectrometer. EI-MS were on a SX102A (JEOL) mass spectrometer. Kieselgel 60 (Merck No, 9385 and 7729) and Sephadex LH-20 (Pharmacia Fine Chemicals) were used for column chromatography. Preparative HPLC was carried out on a DelataPak C18 column (19 mm×300 mm, Waters) with UV detection at 220 nm. Gelatin were purchased from Sigma Chemical Co.. Fetal bovine serum (FBS), Medium 199 (M199) were obtained from GIBCO/BRL Laboratories. 96 well plates were purchased from Corning Costar Co.

Fermentation and Isolation of Compounds

An unidentified fungal strain B90911 isolated from a soil sample collected in the field of Yuseong, Daejeon was cultured in a PD medium for three days. The seed culture was inoculated to a sterilized rice-bran in five 2-liter-flask and continued culturing for a week at 28°C. The solid culture was extracted once with the same volume of acetone (9 liters) and the cell debris was removed by filtration. The filtrate was evaporated to a small volume (0.5 liters) and partitioned between H_2O and EtOAc twice. The organic layer was concentrated to dryness *in vacuo*. The EtOAc soluble fraction (2 g) was chromatographed on a silica gel column eluted with EtOAc/MeOH (5 : 1). The active fraction was subjected to a Sephadex LH-20 column chromatography (MeOH) and further purified by preparative HPLC eluted with a gradient of CH_3CN/H_2O [0′(45 : 55), 15′(60 : 40), 16′(100 : 0), 19′(100 : 0), 20′(45 : 55), flow rate; 1 ml/minute] to give compounds **1** (40 mg), **2** (17 mg), **3** (7.5 mg), and **4** (9 mg).

Cell Lines and Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (Rockville, MD). The HUVECs were plated onto 0.3% gelatin-coated culture dishes and grown in M199 medium supplemented with heat-inactivated 20% (v/v) fetal bovine serum (FBS), 3 ng/ml basic fibroblast growth factor (bFGF), 10 U/ml heparin, 100 units/ml penicillin, and 100 units/ml streptomycin at 37°C incubator in a humidified atmosphere containing 5% CO_2. HUVECs were used between passage 8 and 12.

In Vitro Tube Formation Assay

HUVECs grown in the complete medium were changed

Table 1. ^1H and ^{13}C NMR data of compounds **2**, **3**, and **4** in DMSO-d_6.

Position	2		3		4	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	107.3		108.4		112.5	
2	156.5		152.4		149.2	
3	109.6	6.31 (br s)	113.8		115.6*	
4	141.3		139.5		135.7	
5	104.4	5.63 (br s)	106.4	5.90 (br s)	115.5*	
6	157.1		155.28		149.0	
7	167.1		166.7		163.8	
8	51.9	3.76 (s)	52.3	3.82 (s)	52.1	3.28 (s)
9	21.4	2.06 (s)	20.4	2.16 (s)	18.1	2.45 (s)
1'	125.7		125.4		124.4	
2'	107.5	6.74 (d, 2.9)	107.6	6.77 (br s)	107.4	6.76 (d, 2.8)
3'	133.8		133.4		135.1	
4'	104.8	6.76 (d, 2.9)	104.9	6.77 (br s)	104.7	6.66 (d, 2.8)
5'	153.5		153.3		152.8	
6'	155.2		155.29		154.7	
7'	165.4		165.0		164.5	
8'	52.1	3.61 (s)	52.1	3.62 (s)	51.9	3.65 (s)
9'	56.1	3.69 (s)	56.1	3.70 (s)	56.1	3.61 (s)

δ: ppm from TMS, *: exchangable

to a serum-starvation for overnight in M199 medium supplemented with heat-inactivated 0.5% (v/v) fetal bovine serum (FBS), 3 ng/ml basic fibroblast growth factor (bFGF), 10 U/ml heparin, 100 units/ml penicillin, and 100 units/ml streptomycin. After overnight starvation, the cells were transferred onto 96-well plate coated with Matrigel (Becton Dickinson, Bedford, MA, USA) at 2×10^4 cells/well in M199 medium supplemented with heat-inactivated 0.5% (v/v) fetal bovine serum (FBS), 10 U/ml heparin, 100 units/ml penicillin, and 100 units/ml streptomycin. Recombinant human VEGF (10 ng/ml) was added with various concentrations of compounds tested. Radicicol was used as a positive compound[3]. Cells were incubated at 37°C for 16 hours to 48 hours and morphological changes of HUVECs were observed and photographed three different fields per well under a microscope (×100)[4]. Inhibition of tube formation was assessed by the measurement of total tube-length formed in each photograph using Adobe Photoshop.

Results and Discussion

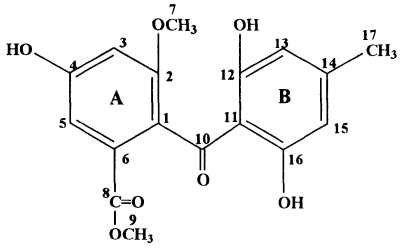
Structure Elucidation

HRFAB-MS spectrum of compound **1** had a molecular ion peak at *m/z* 333.0975 $[M+H]^+$ indicating that the molecular formula is $C_{17}H_{17}O_7$. The ^1H NMR spectrum of **1** indicated the presence of four aromatic protons, one methyl group, two methoxyl groups, and three hydroxyl protons. Thus the structure of compound **1** was identified to be sulochrin by comparison of their physical and spectral data with those of previous report[5].

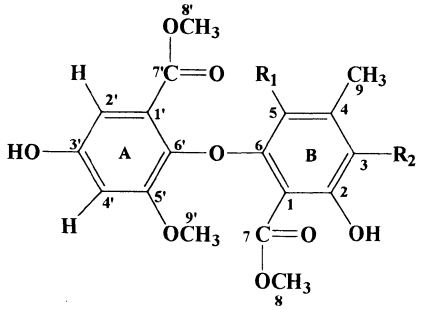
Compound **1** (sulochrin)–pale yellow needle, mp: 240~245°C negative ESI-MS (*m/z*): 331 $[M-H]^+$, High resolution FAB-MS (*m/z*): 333.0975 $[M+H]^+$ (calculated for $C_{17}H_{17}O_7$: 333.0974), UV λ_{max} MeOH nm: 286, 209, ^1H-NMR (DMSO-d_6, 300 MHz) δ: 2.15 (3H, br s, 17-CH_3), 3.64 (3H, s, 9-OCH_3), 3.65 (3H, s, 7-OCH_3), 6.09 (2H, s, 13-, 15-H), 6.68 (1H, d, J=1.8 Hz, 3-H), 6.91 (1H, d, J=2.1 Hz, 5-H), 9.98 (1H, br s, 4-OH), 11.44 (2H, br s, 12-, 16-OH), ^{13}C-NMR (DMSO-d_6, 75.5 MHz) δ: 126.2 (C1), 156.8 (C2), 103.4 (C3), 158.1 (C4), 107.2 (C5), 127.9 (C6), 56.0 (C7), 165.7 (C8), 52.0 (C9), 199.6 (C10), 109.2 (C11), 161.7 (C12, C16), 107.6 (C13, C15), 147.4 (C14), 21.6 (C17).

The molecular formula of compound **2** was determined to be $C_{18}H_8O_8$ from the positive HR-FABMS spectrum (*m/z*; found 363.1087 $[M+H]^+$; calcd 363.1080). The UV absorption maxima at 258 and 331 nm in MeOH resembled those of asterric acid derivatives, which possess the biphenyl ether structure[6,7]. This compound, biosynthetically formed from sulochrin *via* bisdechlorogeodin[8] was previously reported[7].

Fig. 1. Chemical structures of compounds isolated from an unidentified fungal strain B90911.



Sulochrin (**1**)



methyl asterric acid (**2**) : R_1=R_2=H

methyl 3-chloroasterric acid (**3**): R_1=H, R_2=Cl

methyl 3,5-dichloroasterric acid (**4**) : R_1=Cl, R_2=Cl

The ^1H and ^{13}C NMR spectra of **3** were essentially identical to those of **2** except for the lack of an aromatic methine proton and down field shift of one aromatic carbon signal. The FAB-MS spectrum of **3** showed $[M+Na]^+$ ion at *m/z* 419, 421 with a peak intensity ratio of approximately 3 : 1 to the $[M+Na]^+$ +2 peak, indicating the presence of one chlorine atom. This was confirmed by the molecular formula of $C_{18}H_{17}ClO_8$ from the positive HR-FABMS spectrum (*m/z*; found 397.0683 $[M+H]^+$; calcd 397.0692). These data indicated that compound **3** have the same structure as **2** except a substitution of one aromatic proton by a chlorine atom. The proper positioning of chlorine group was established by HMBC experiments. HMBC correlations of a methine proton at δ 5.90 (1H, br s) with carbons at δ 20.4 (C-9), 108.4 (C-1), 113.8 (C-3), and

155.28 (C-6) indicated the placement of the chlorine at C-3.

The ^{1}H and ^{13}C NMR spectra of **4** were very similar to those of **2** except missing two aromatic methine protons and down field shift of two aromatic carbon signals. The molecular formula of **4** was determined to be $C_{18}H_{16}Cl_2O_8$ from the positive HR-FABMS spectrum (*m/z*; found 431.0299 $[M+H]^+$; calcd 431.0301). These data indicated that compound **4** is 3,5-dichloride derivative of compound **2**.

Based on the above the spectral data compound **3** and **4** were determined to be 3-chloro- and 3,5-dichloroasterric acid, new fungal metabolites in nature.

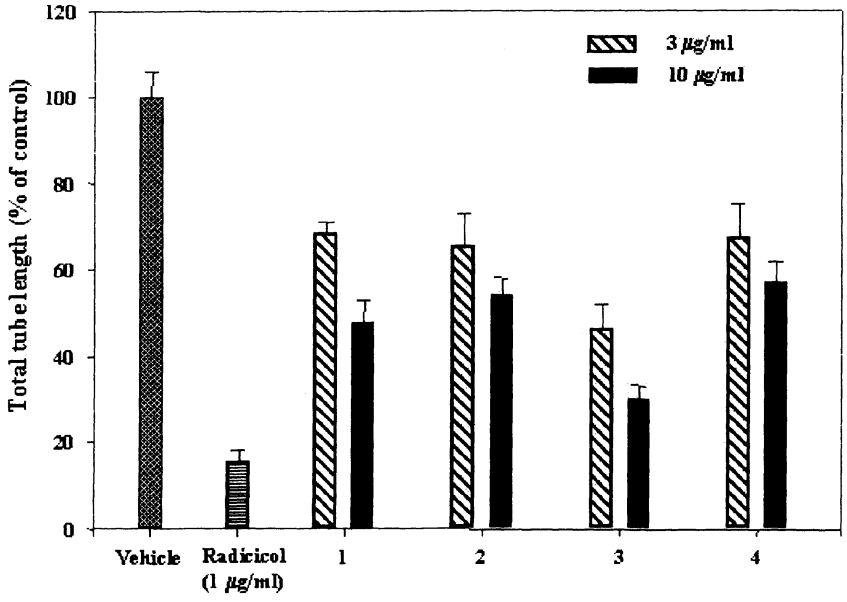
Effects of Compounds **1**~**4** on the VEGF-induced Tube Formation of HUVECs

To determine the inhibitory effect of the compounds **1**~**4** on the angiogenesis *in vitro*, HUVECs were used as a model on the basis that they migrate and differentiate into capillary-like tubes on Matrigel. This differentiation process of endothelial cells is considered to be one of the key steps in angiogenesis.

Compounds **1**~**4** were independently added at various concentrations into the wells along with VEGF (10 μg/ml), which is one of the key promoters of tumor induced angiogenesis, and the plate was incubated for 16~48 hours in a CO_2 incubator. The formation of capillary-like tube was monitored under a microscope during the incubation. Inhibition of tube formation was assessed by the measurement of total tube-length in comparison with vehicle treated control. The cells in the well containing vehicle (DMSO) became to be more robust and longer hollow tube networks as the incubation proceeded, but the tubes in the wells containing compound **1**~**4** were less extensive, thinner, and foreshortened when compared with DMSO controls. All compounds tested inhibited VEGF-induced tube formation of HUVECs by 30~70% at the concentration of 10~3 μg/ml. Treatment with 10 μg/ml of compound **3** resulted in a strong inhibition of tube formation by 70%. A quantitative analysis revealed that 3-chloroasterric acid (**3**) inhibited VEGF-induced tube formation of HUVECs more potently than the other compounds, however, its potency much weaker than that of 1 μg/ml of radicicol (Fig. 2).

Sulochrin and its derivatives, known fungal metabolites, have very weak antibacterial, antifungal, and eosinophil

Fig. 2. Effects of compounds **1**~**4** on *in vitro* angiogenesis.



HUVECs were seeded with or without compounds tested on polymerized Matrigel as described in material and method. Radicicol was used as a positive control. Changes of cell morphology were captured through inverted microscope (×100) and photographed three different fields per well by Image-Pro Plus. Inhibition of tube formation was assessed by the measurement of total tube-length formed in each photograph using Adobe Photoshop. Each bar represents the average±S.D. of two individual wells.

degranulation inhibitory activities[5,9]. Asterric acid, a common skeleton of the isolated compounds has been reported to inhibit endothelin binding *in vitro*[6,7]. Our study demonstrates for the first time that the compound having asterric skeleton show anti-angiogenic activity.

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