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COMMUNICATIONS TO THE EDITOR

A Novel Dihydroxanthenone, AGI-B4 with Inhibition of VEGF-induced Endothelial Cell Growth

Sir:

Vascular endothelial cell growth factor (VEGF) was first discovered as a tumor-secreted protein that induced a transient and reversible hyperpermeability.[1] VEGF was subsequently recognized as a potent mitogen that stimulates both growth and migration of vascular endothelial cells.[2] The recognition of VEGF as one of the primary stimulants of angiogenesis has led to the development of neutralizing antibodies,[3] soluble receptor constructs,[4] antisense strategies,[5] and synthetic inhibitor of receptor tyrosine kinase[6] that block angiogenesis or suppress tumor growth by interfering with VEGF signaling.

In the screening of anti-angiogenic substances inhibiting VEGF-induced proliferation of human umbilical vein endothelial cells (HUVECs), we isolated a new compound 7,8-dihydroxanthenone-8-carboxylic acid methyl ester (**1**) from an *Aspergillus* sp. Y80118 by bioassay-guided fractionation and isolation. In this communication, we describe the isolation and structure determination of compound **1** and its inhibition of VEGF-induced HUVECs proliferation.

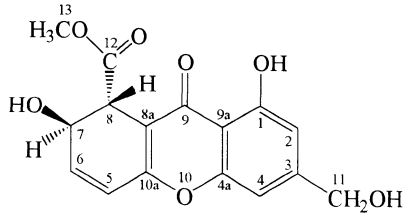
The producing organism, *Aspergillus* sp. Y80118 was isolated from a soil sample collected in Gongju, Korea and has been deposited at the Korea Collection for Type Culture (KCTC) as an accession number of KCTC 0737BP. A slant culture of the strain Y80118 grown on malt extract agar was inoculated into an 1 liter baffled flask containing 300 ml of culture medium consisting of glucose 2%, yeast extract 0.2%, polypeptone 0.5%, magnesium sulfate 0.05%, and KH_2PO_4 0.1% (pH 5.6~5.8 before sterilization). The flask was incubated at 27°C for 6 days on a rotary shaker (150 rpm). The inhibitory activity of VEGF-induced HUVEC proliferation reached a maximum at 6 days culture. After the fermentation, the culture broth (14 liters) was extracted with an equal volume of acetone and then the mixture was filtered. The filtrate was concentrated *in vacuo* to a small volume and the residue was extracted with EtOAc. The EtOAc extract (5.4 g) was chromatographed on a silica gel column (Kieselgel 60, Merck) eluted with a CH_2Cl_2-MeOH step gradient system [CH_2Cl_2, CH_2Cl_2-MeOH (50:1, 20:1), MeOH, each 1 liter] to obtain 4 fractions. The active fraction, which was eluted with the solvent ratio at 20:1, was concentrated *in vacuo*. The residue (2.6 g) was rechromatographed on a silica gel column eluted with a $CHCl_3$-MeOH step gradient system [$CHCl_3$, $CHCl_3$-MeOH (40:1, 20:1, 10:1, 5:1, 1:1), MeOH, each 300 ml] to obtain 22 fractions. The active fractions were concentrated *in vacuo* to give a yellow powder (350 mg). Compound **1** was finally purified with preparative TLC (Kieselgel 60, Merck) developed with $CHCl_3$-mixed solvent [$CHCl_3$-MeOH-acetic acid-H_2O (68:20:10:2)] (65:35) to afford 130 mg of **1**. Other related compounds such as sydowinin A (27 mg) and B (**2**, 19 mg) and 1-hydroxy-3-hydroxymethyl-7,8-epoxy-xanthenone-8-carboxylic acid methyl ester (1.2 mg) were also purified.[7,8]

Compound **1** was a yellow powder and had a mp of 166~168°C and $[\alpha]_D^{25}$ of −1.6° (*c* 0.4, MeOH). The molecular formula was established as $C_{16}H_{14}O_7$ by HRFAB-MS (*m/z* $[M+H]^+$; calcd. 319.0818, found 319.0813). It gave positive responses to the iodine, sulfuric acid, and ferric chloride tests. The UV absorption pattern of **1** at 215 nm (log ε 4.41), 271 nm (log ε 4.45) and 343 nm (log ε 3.73) was very similar to that of sydowinol isolated together with sydowinin A and B from *Aspergillus sydowi*.[7] The UV pattern resembled that of MS-347a isolated together with sydowinin B (**2**),[8] suggesting that compound **1** has a dihydroxanthenone skeleton. The absorption at 3420 cm^{-1}, 1735 cm^{-1}, and 1652 cm^{-1} in the IR spectrum of **1** indicated the presence of hydroxyl, ester, and chelated carbonyl moieties, respectively. The ^{13}C NMR and DEPT spectra revealed sixteen carbon signals composed of one methyl, one of each oxygenated-methylene and -methine, one aliphatic methine, four aromatic methines, six aromatic quaternary carbons, and two carbonyl carbons. The ^{13}C NMR signals at δ 172.8 and 182.5 were assigned to ester and chelated carbonyl carbons, respectively (Table 1). The ^1H NMR spectrum of **1** in acetone-d_6 revealed four aromatic protons at δ 7.01 (br s), 6.78 (br s), 6.66 (dd, J=9.9, 4.8 Hz), and 6.52 (d, J=9.9 Hz). The two broad singlets at δ 7.01 and δ 6.78 were suggestive of weak *meta*-coupling. Two *ortho*-coupled aromatic protons at δ 6.66 (dd, J=9.9, 4.8 Hz), and 6.52 (d, J=9.9 Hz) were connected to those at δ 4.71 (dd. J=4.8, 3.6 Hz) and 4.15 (d, J=3.6 Hz). This connectivity was

Table 1. ^{1}H and ^{13}C NMR data of AGI-B4 in acetone-d_6.

Position	δ_C (70 MHz)	δ_H (300 MHz)
1	161.5	-
2	109.7	6.78 (1H, br s)
3	152.5	-
4	105.7	7.01 (1H, br s)
4a	157.1	-
5	123.2	6.52 (1H, d, J=9.9Hz)
6	140.6	6.66 (1H, dd, J=9.9, 4.8Hz)
7	65.8	4.71 (1H, dd, J=4.8, 3.6Hz)
8	46.2	4.15 (1H, d, J=3.6Hz)
8a	111.6	-
9	182.5	-
9a	110.4	-
10a	160.8	-
11	64.3	4.67 (2H, s)
12	172.8	-
13	53.2	3.70 (3H, s)

Fig. 1. Chemical structures of AGI-B4 (**1**) and sydowinin B (**2**).



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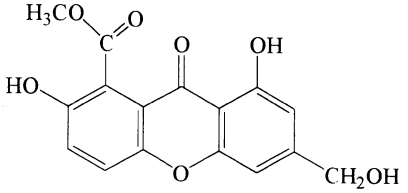

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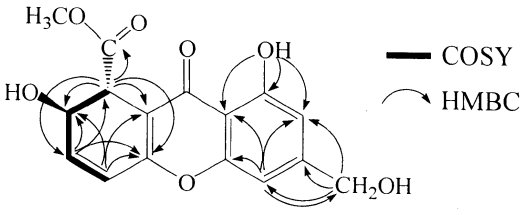
Fig. 2. ^{1}H-^{1}H correlation in the COSY spectrum and ^{1}H-^{13}C correlation in the HMBC spectrum of AGI-B4.



confirmed by correlation of these four proton signals in the ^{1}H-^{1}H COSY spectrum (Fig. 2). In addition, one methoxyl and one hydroxymethyl protons appeared at δ 3.70 (3H, s) and 4.67 (2H, s), respectively and a hydrogen-bonded hydroxyl proton was observed at δ 12.51. The precise connectivities between proton and carbon signals were established by interpretation of HMBC data. In the HMBC spectrum of **1**, the proton at δ 4.15 was correlated with C-6 (δ 140.6), -7 (65.8), -8a (111.6), -10a (160.8), and -12 (172.8) (Fig. 2). These correlations further indicated that the methoxycarbonyl group was connected to C-8. On the basis of these spectroscopic analyses, the structure of compound **1** was determined to be a new compound, 1,7-dihydroxy-3-hydroxymethyl-7,8-dihydroxanthenone-8-carboxylic acid methyl ester, which is a dihydro form of sydowinin B and named AGI-B4. Several xanthenones and dihydroxanthenones have been isolated from *Aspergillus* sp. and *Penicillium* sp. Among dihydroxanthenones, sydowinol and F390C possess identical molecular formula to the compound **1**, but the positions of hydroxyl and methoxycarbonyl groups in them are different from those of compound **1**.[7,9] In order to determine the relative stereochemistry of **1**, an X-ray structure analysis was undertaken with yellow crystals grown from methanolic solution. The crystal data as follows: Empirical formula; $C_{16}H_{14}O_7$. Formula weight; 318.08. Crystal system; triclinic. Lattice parameters; a=8.079(1) Å, b=9.824(1) Å, c=10.226(1) Å, α=67.00(1)°, β=68.90(1)°, γ=70.76(1)°, V=680.0(1) Å^3. Space group; $P\bar{1}$ (bar). Z value; 2. D_{calc}; 1.554 g/cm^3. Intensity data were collected by the $\omega/2\theta$ scan technique with the range of $3<2\theta<140°$ from a 0.2×0.3×0.2 mm sized crystal on a KAPPA goniometer (MAC Science) using CuKα radiation generated by a rotating-anode (50 kV, 90 mA). A total of 2687 reflections were measured, and 164 reflections out of 2575

Fig. 3. Perspective view of the crystal structure of AGI-B4.

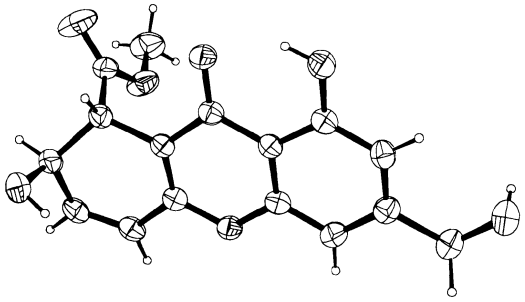
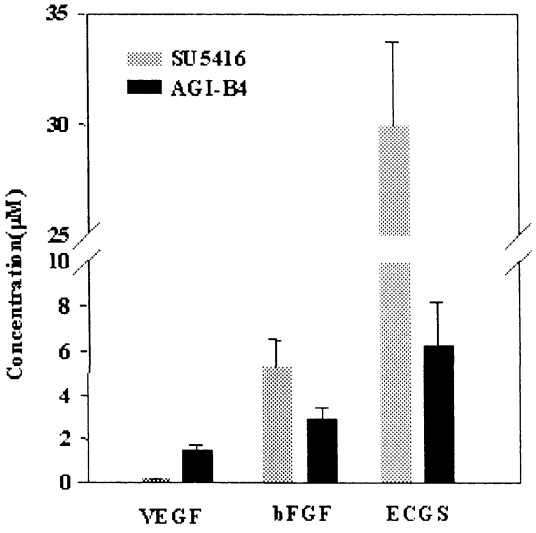


Fig. 4. Effect of AGI-B4 (**1**) and SU5416 on the proliferation of HUVECs induced by VEGF, bFGF, or ECGS as expressed by IC_{50} values.



HUVECs (passage 7) starved overnight in M199 medium containing 2.5% FBS were plated at a density of 1×10^4 cells/ml in a gelatin-coated 96-well plate with 200 μl of M199 medium containing 2.5% FBS and 10 unit of heparin. After 4 hours, VEGF (10 ng/ml) or bFGF (10 ng/ml) and various concentrations of compound **1** or SU5416 were added. After 2 days, cell proliferation was measured by acid phosphatase assay[15]. As a control experiment HUVECs were incubated in the M199 medium containing 10% FBS supplemented with ECGS (0.05 mg/ml) and various concentration of compound **1** or SU5416. Each sample was assayed in triplicate and the independent experiment was repeated twice.

independent reflections with $I>2\sigma(I)$ were treated as unobserved. The structure was solved by direct method with SIR92[10] incorporated in maXus1.1.[11] All the 23 non-hydrogens with anisotropic thermal parameters and 14 hydrogens with isotropic ones were refined by the full matrix least-squares procedure to the final R-value of 0.046 for 2411 observed reflections. The molecular structure of **1** is illustrated in Fig. 3, therefore, the relative structure of AGI-B4 was confirmed as shown in Fig. 1.

The compound **1** is structurally similar to sydowinin A and sydowinin B.[7,8] In particular, MS-347a, reported together with sydowinin B, having an epoxide group instead of allylic alcohol in compound **1**, is known to have inhibitory activities against smooth muscle myosin light chain kinase and protein kinase C.[8] Meanwhile three dihydroxanthenones, nidulain A[12] and F390B and C[9] were reported as inhibitors of DNA topoisomerases[13] and of tumor cell growth.[9]

Since the compound **1** was isolated by its inhibition of VEGF-induced HUVEC proliferation, we compared the effect of **1** on the proliferation of HUVECs under different stimuli such as VEGF, bFGF, or endothelial cell growth supplement (ECGS, Sigma 3149). As shown in Fig. 4, **1** inhibited the proliferation of HUVECs induced by VEGF, bFGF or ECGS with IC_{50} of 1.4 μM, 2.8 μM, and 6.2 μM, respectively. Meanwhile, SU5416, a selective inhibitor of VEGF receptor[6] inhibited the proliferation of HUVECs induced by VEGF, bFGF, or ECGS with IC_{50} of 0.05 μM, 5.3 μM, and 30.5 μM, respectively. These results indicated that the compound **1** inhibited VEGF-induced HUVEC proliferation with marginal selectivity, however, much weaker than that of SU5416. Since compound **1** is a novel inhibitor of VEGF signaling, which is one of the primary stimulants of angiogenesis, it would be useful in elucidating the molecular mechanism of angiogenesis as well as in developing anti-angiogenic agents for the treatment of angiogenesis-associated diseases such as tumor and rheumatoid arthritis.[14] Further study is required to define the mechanism of anti-angiogenic activity of compound **1**.

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