Communications to the Editor

NEW ANGIOGENESIS INHIBITORS, WF-16775 A_1 AND A_2

Sir:

Angiogenesis, which is the process of new blood vessel formation, is associated with various diseases, such as diabetic retinopathy, rheumatoid arthritis and solid tumors[1]. Thus it is expected that angiogenesis inhibitors which prevent neovascularization would have an applicability as a therapy for these diseases. During our studies on the screening program for new angiogenesis inhibitors, we found that a fungus *Chaetasbolisia erysiophoides* No. 16775 produced new angiogenesis inhibitors, WF-16775 A_1 and A_2. In this communication we describe isolation, characterization, structural elucidation and biological properties of WF-16775 A_1 and A_2.

The WF-16775 A_1 and A_2 producing strain, *Chaetasbolisia erysiphoides*, which was originally isolated from a soil sample collected at Mt. Hakusan, Ishikawa Prefecture, Japan, has been deposited in Fermentation Research Institute, Agency of Industrial Science as FERM P-11873. The strain was cultured at 25°C for 7 days in 150 liters of a production medium consisting of soluble starch 2%, sucrose 0.2%, chicken meat bone meal 1%, dried yeast 0.5%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.2%, $ZnSO_4 \cdot 7H_2O$ 0.1% and $CaCO_3$ 0.2%.

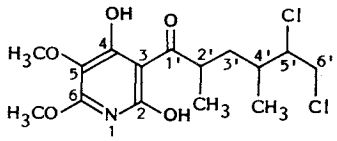
The fermentation broth (150 liters) was filtered and the mycelial cake was soaked twice with 80% aqueous acetone (20 liters). The aqueous solution after removal of acetone was adjusted to pH 4 with 6 N HCl and extracted twice with ethyl acetate (10 liters). The ethyl acetate layer was separated and concentrated *in vacuo* to give an oily material (570 g). The oily material was mixed with silica gel (1 liter) and the resultant dry powder was applied on a silica gel chromatographic column (2 liters) packed in *n*-hexane. After developing with *n*-hexane (9 liters), the column was eluted stepwisely with each time three column volumes of a mixture of *n*-hexane and ethyl acetate (5 : 1 and 2 : 1). The active fractions were combined and concentrated *in vacuo* to give an oily residue (34 g). The resultant precipitate was diluted with methanol (340 ml) whereafter distilled water (510 ml) was added and the pH adjusted to 7.5 with 1 N NaOH. The solution which contained two active fractions, was applied on an octadecyl substituted silica (ODS) gel column (1 liter) packed in 40% aqueous methanol containing 10 mM potassium phosphate buffer, pH 7. After washing the column with the above solvent, the column was eluted with 45% aqueous methanol (WF-16775 A_1) and then 50% aqueous methanol containing 10 mM potassium phosphate buffer, pH 7 (WF-16775 A_2), respectively. The active materials were concentrated *in vacuo* to remove methanol. Extractions with *n*-hexane at pH 7 gave pale yellow extracts from which 83 mg of WF-16775 A_1 and 273 mg of WF-16775 A_2 were obtained as white crystalline needles after concentration.

WF-16775 A_1 and A_2 were readily soluble in

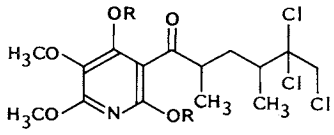
Table 1. Physico-chemical properties of WF-16775 A_1 and A_2.

	WF-16775 A_1	WF-16775 A_2
Appearance	Colorless needles	Colorless needles
MP	85.0~86.5°C	119.5~121.0°C
$[\alpha]_D^{20}$	−7° (*c* 1.0, $CHCl_3$)	−52° (*c* 0.5, $CHCl_3$)
Molecular formula	$C_{15}H_{21}Cl_2NO_5$	$C_{15}H_{20}Cl_3NO_5$
Mass spectrum		
FAB-MS (*m/z*)	366 $(M+H)^+$	400 $(M+H)^+$
HRFAB-MS Found:		400.0488
Calcd:		400.0485
Elementary analysis		
Found:	C 49.58, H 5.78, N 3.91, Cl 19.79	C 45.13, H 4.97, N 3.49, Cl 26.67
Calcd:	C 49.18, H 5.78, N 3.82, Cl 19.38	C 44.96, H 5.03, N 3.50, Cl 26.55
UV λ_{max}^{MeOH} nm (ε)	236 (16,800), 269 (11,300), 318 (9,500)	236 (19,200), 269 (12,400), 322 (11,600)
IR ν_{max}^{KBr} cm^{-1}	1650, 1597, 1443, 1326, 1197, 1162, 993	1644, 1610, 1456, 1333, 1207, 1169, 995

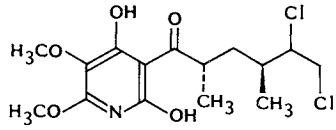
Fig. 1. Structure of WF-16775 A_1 (**1**), A_2 (**2**) and atpenin A_5.



1



2 R=H
2a R=$COCH_3$



Atpenin A_5

methanol and ethyl acetate, and insoluble in water. These compounds gave positive reactions to ferric chloride and iodine vapor, though negative to Molish and ninhydrin reagents. Physico-chemical properties of WF-16775 A_1 and A_2 are summarized in Table 1. Further, initial structural efforts were carried out on WF-16775 A_2 (**2**) because WF-16775 A_2 was more abundant component (Fig. 1).

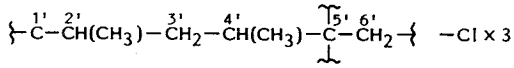
HRFAB-MS measurement of WF-16775 A_2 (**2**) (found 400.0488, calcd for $C_{15}H_{20}Cl_3NO_5$ 400.0485) yielded a molecular formula of $C_{15}H_{20}Cl_3NO_5$ for **2** which was in good agreement with elementary analysis (Table 1).

A combination of ^{1}H-^{1}H COSY and ^{13}C-^{1}H COSY revealed the following fragments:

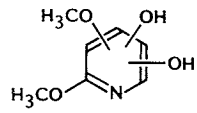
$$-\overset{2'}{C}H(CH_3)\overset{3'}{C}H_2\overset{4'}{C}H(CH_3)-;\ -\overset{6'}{C}H_2-;\ -OCH_3\times 2\,.$$

In COLOC spectrum, a ketone carbonyl carbon at δ 210.9 was correlated with 2′-H (4.03) and a quaternary carbon at δ 97.9 was correlated with methyl protons (1.17) on C-4′ and with isolated methylene protons (4.18 and 4.16). These long-range ^{13}C-^{1}H coupling patterns extended the above fragment to partial structure **i** (Fig. 2). Methoxy protons (3.99) were long-range coupled to sp^2 quaternary carbon at δ 160.0 and methoxy protons (3.72) to sp^2 quaternary carbon at δ 124.7. Acetylation of **2** afforded diacetyl derivative **2a** in high yield. The ^{1}H chemical shifts of the two acetyl methyl (2.32 and 2.29) and strong IR absorption band at 1780 cm^{-1} are quite characteristic of phenol acetate. This fact indicated that **2** possessed two phenolic OH groups. In conjunction with these, the remaining four double bond equivalent and one

Fig. 2. Partial structures of WF-16775 A_2.



i



ii

nitrogen atom assumed the presence of pyridine nucleus substituted with $-OCH_3\times 2$ and $-OH\times 2$ (**ii**) in Fig. 2. This is only one reasonable combination between the partial structures shown in Fig. 2 and thus the structure of **2** was elucidated without clarification of pyridine substitution pattern. Finally the pyridine substitution pattern of **2** was elucidated by comparison of the ^{13}C NMR data with that of WF-16775 A_1 (**1**) (Fig. 1, and Tables 2 and 3).

The molecular formula of WF-16775 A_1 (**1**) was established as $C_{15}H_{21}Cl_2NO_5$ based on FAB-MS and elementary analysis (Table 1). From a CAS registry search it came to our attention that WF-16775 A_1 (**1**) might be identical with atpenin A_5[2]. The physico-chemical properties of **1** (Table 1) closely matched those of atpenin A_5[2]. However, ^{13}C and ^{1}H NMR data of **1** in CD_3OD (Table 2) are fairly different from those reported for atpenin A_5 in $CDCl_3$[2]. In the same D-solvent ($CDCl_3$), ^{13}C and ^{1}H NMR spectral data (Table 2) are in excellent agreement with those of atpenin A_5. This fact

Table 2. ^1H and ^{13}C NMR data of WF-16775 A_1.

	^1H (400 MHz)		^{13}C (100 MHz)	
Position	δ^a multiplicity	δ^b multiplicity	δ^a	δ^b
2			166.6	161.8
3			100.8	100.6
4			162.6	Missing
5			124.7	Missing
6			160.1	155.5
5-OCH_3	3.70 (3H, s)	3.80 (3H, s)	61.2	61.6
6-OCH_3	4.00 (3H, s)	4.20 (3H, s)	55.7	58.3
1′			211.3	209.8
2′	4.14 (m)	4.20 (m)	41.7	39.5
3′	1.87 (ddd, 13.5, 7.5, 7.5)	1.90 (m)	39.2	37.6
	1.47 (m)	1.50 (m)		
4′	2.18 (m)	2.18 (m)	34.2	32.6
5′	4.15 (m)	4.13 (m)	67.3	65.5
6′	3.76 (d, 12)	3.72 (dd, 11.5, 6)	47.1	45.9
	3.72 (d, 12)	3.64 (dd, 11.5, 8.5)		
2′-CH_3	1.15 (3H, d, 6.5)	1.16 (3H, d, 6.5)	17.9	18.0
4′-CH_3	0.93 (3H, d, 6.5)	0.93 (3H, d, 6.5)	13.2	12.9

[a] In CD_3OD.
[b] In $CDCl_3$.
Coupling constants (J in Hz) are shown in parentheses.

Table 3. ^1H and ^{13}C NMR data of WF-16775 A_2

	^1H (400 MHz)		^{13}C (100 MHz)	
Position	δ^a multiplicity	δ^b multiplicity	δ^a	δ^b
2			166.6	161.4
3			100.5	100.2
4			162.5	Missing
5			124.7	121.8
6			160.0	155.5
5-OCH_3	3.72 (3H, s)	3.81 (3H, s)	61.5	61.6
6-OCH_3	3.99 (3H, s)	4.20 (3H, s)	55.9	57.9
1′			210.9	209.5
2′	4.03 (m)	4.08 (m)	42.4	40.8
3′	1.80 (m)	1.84 (m)	36.1	34.4
	1.77 (m)	1.78 (m)		
4′	2.51 (m)	2.50 (m)	43.2	41.8
5′			97.9	96.4
6′	4.18 (d, 12)	4.13 (d, 12)	54.1	53.1
	4.16 (d, 12)	4.10 (d, 12)		
2′-CH_3	1.14 (3H, d, 6.5)	1.18 (3H, d, 6.5)	16.8	16.5
4′-CH_3	1.17 (3H, d, 6.5)	1.22 (3H, d, 6.5)	14.9	14.5

[a] In CD_3OD.
[b] In $CDCl_3$
Coupling constants (J in Hz) are shown in parentheses.

suggested that **1** had the same relative stereochemistry as atpenin A_5. The absolute stereochemistry remains undefined as the solvent used in specific rotation of **1** was different from that of atpenin A_5.

The effects of WF-16775 A_1 and A_2 on the proliferation of *in vitro* cultured human umbilical vein endothelial (HUVE) cells, mouse lymphoma EL-4 cells and mouse fibrosarcoma MethA cells were examined[3]. The concentration of WF-16775 A_1 required for 50% of cell growth for HUVE, EL-4 and MethA cells were 0.16, 0.16 and 0.16

Table 4. Inhibitory effects of WF-16775 A_1 and A_2 on angiogenesis in chorioallantoic membranes (CAMs).

Dose (μg/pellet)	Number of CAM assayed	Number of CAM with following capillary density[a]		
		Normal	Lower	Avascular
WF-16775 A_1				
0.1	15	12	1	2
0.5	19	4	4	11
2.5	16	2	2	12
5.0	7 (4/11: Tox)	0	1	6
WF-16775 A_2				
0.05	20	11	8	1
0.1	26	7	11	8
0.5	21	1	6	14
2.5	23	1	2	20
5.0	8 (3/11: Tox)	0	0	8

[a] Density of capillaries developed around the pellet.

μg/ml, respectively. Further, IC_{50} value of A_2 for the cells described above were 0.02, 0.80 and 0.64 μg/ml, respectively. A_2 was the most effective against HUVE cells at low concentration, though this effect was cytostatic.

The inhibitory effects of WF-16775 A_1 and A_2 on angiogenesis in chick embryo chorioallantoic membrane (CAM) were examined by the method of TANAKA *et al.* with a slight modification[4]. At least 15 fertilized eggs were used for each doses of these compounds. The antiangiogenic response was evaluated by measuring an avascular zone in the CAM around the pellet according to the method of CRUM *et al.*[5]. The results are shown in Table 4. Compared to the empty pellet without samples, WF-16775 A_1 at doses of 0.5~2.5 μg/pellet and A_2 at doses of 0.1~2.5 μg/pellet displayed the potent antiangiogenic activity.

These results suggest that WF-16775 A_1 and(or) A_2 will be a promising candidate for the angiogenesis dependent diseases. Further studies on angiogenesis inhibitory activities of these compounds are in progress.

TAKANAO OTSUKA
SHIGEHIRO TAKASE
HIROSHI TERANO
MASAKUNI OKUHARA

Exploratory Research Laboratories,
Fujisawa Pharmaceutical Co., Ltd.,
5-2-3 Tokodai, Tsukuba,
Ibaraki 300-26,
Japan

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