Phoenistatin, A New Gene Expression-Enhancing Substance Produced by *Acremonium fusigerum*

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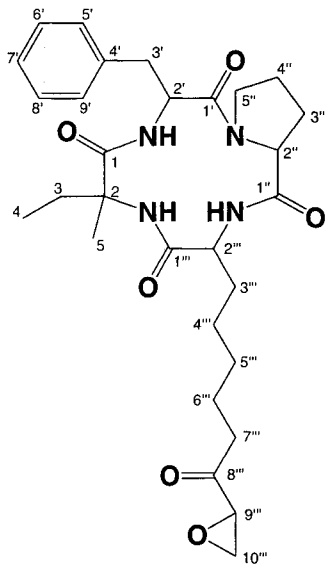
A variety of growth factors and cytokines control proliferation and differentiation and cell survival in various types of cells by regulating gene expression. Transforming growth factor-β (TGF-β), first discovered as a trophic factor for several different cell lines,[1] is now well known to act as a growth inhibitor against various kinds of mammalian cells[2]. The mode of action of TGF-β is not fully understood but it is known to induce expression of a variety of genes such as a tumor suppression gene *p21*, plasminogen activator inhibitor-1 (PAI-1), to suppress tumor progression. Transfection with a TGF-β type II receptor gene into SW48 colon cancer cells, which lack functional cell surface TGF-β type II receptors, restores the susceptibility to growth inhibition by TGF-β and reverses the *in vitro* and *in vivo* malignant phenotype[3]. In addition, the restoration of TGF-β signaling in LNCaP human prostate cancer cells suppresses tumorigenicity with inducing apoptosis[4]. Thus, substances which mimic the gene expression-enhancing activity of TGF-β are expected to be useful chemotherapeutic agents for cancer treatment. In order to detect the gene expression ability of TGF-β, we constructed an assay system utilizing the expression of a reporter gene. Mv1Lu mink lung epithelial cells express TGF-β receptors abundantly and respond to TGF-β resulting in the expression of plasminogen activator inhibitor-1 (PAI-1)[5]. The cells were transfected with a plasmid bearing the firefly luciferase reporter gene inserted downstream of PAI-1 promoter[6]. Thus microbial samples with TGF-β-like activity can be detected by the production of luciferase which is measured with a luminometer. During the course of our screening using this system, we isolated histone deacetylase inhibitors such as trichostatin A[7,8] and diheteropeptin[9]. Further investigation has resulted in the isolation of a new metabolite with gene expression-enhancing activity, phoenistatin (**1**, Fig. 1). We report herein the production, isolation and structure determination of **1**.

The fungal strain QN5320 was isolated from a dead wood collected at Chichijima Island, Ogasawara, Japan. For the production of **1**, the fungi was inoculated into a seed medium consisting of glucose 1.0%, potato starch 2.0%, polypepton 0.5%, yeast extract 0.5% and $CaCO_3$ 0.4% (pH 7.0), and cultured on a rotary shaker (200 rpm) at 24°C for 3 days. The seed culture was transferred into 500-ml Erlenmeyer flasks containing 100 ml of a production medium composed of glycerol 3.0%, Lab-lemco powder (OXOID) 3.0%, $CaCO_3$ 0.3%, K_2HPO_4 0.05%, KCl 0.03% and $MgSO_4 \cdot 7H_2O$ 0.05% (pH 6.5). Fermentation was carried out on a rotary shaker (200 rpm) at 24°C for 4 days.

The active principle was extracted with ethyl acetate from the supernatant of broth (10 liters). The solvent layer was dried over Na_2SO_4 and concentrated to give an oily

Fig. 1. Structure of phoenistatin (**1**).



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residue. This materiel was subjected to silica gel column chromatography ($CHCl_3$-MeOH=50:1) and successively Sephadex LH-20 column chromatography ($CHCl_3$-MeOH=1:1). A pure sample of **1** (6 mg) was finally obtained by HPLC using a PEGASIL ODS column (20 i. d.×250 mm) with 60% MeOH.

The physico-chemical properties of **1** are summarized in Table 1. The molecular formula of **1** was established as $C_{29}H_{40}N_4O_6$ by high resolution FAB-MS. IR absorptions at 3400, 1680 and 1660 cm^{-1} implied the presence of amide functions. Together with the IR absorptions, four carbonyl carbons observed in the ^{13}C NMR (171.8, 172.8, 174.2 and 175.6 ppm) were assigned to amide carbons in a peptide skeleton. The ^{13}C and ^1H NMR spectral data in $CDCl_3$ are shown in Table 2.

Table 1. Physico-chemical properties of **1**.

Appearance	White powder
MP	60 ~ 62°C
$[\alpha]_D^{23}$	−62.4° (*c* 0.062, MeOH)
Molecular formula	$C_{29}H_{40}N_4O_6$
HRFAB-MS (*m/z*)	
Found	541.3042 $(M+H)^+$
Calcd	541.3026
UV λ_{max}^{MeOH} nm (ε)	203 (12,500), 236 (sh, 2,500)
IR ν_{max} (KBr) cm^{-1}	3400, 1680, 1660, 1520

Table 2. ^{13}C and ^1H NMR data of **1** in $CDCl_3$.

	No.	δ_C	δ_H (multiplicity, *J* = Hz)
+Iva	1	175.6	
	2	63.1	
	3	27.9	2.14 (m), 2.30 (m)
	4	8.4	0.81 (3H, t, *J* = 7.0)
	5	22.4	1.26 (3H, s)
	NH		5.77 (s)
Phe	1'	172.8	
	2'	53.3	5.16 (dt, *J* = 6.0, 10.0)
	3'	35.8	2.94 (dd, *J* = 6.0, 14.0), 3.21 (dd, *J* = 6.0, 14.0)
	4'	137.1	
	5', 9'	129.1	7.20 (2H, d, *J* = 7.0)
	6', 8'	128.6	7.26 (2H, t, *J* = 7.0)
	7'	126.7	7.19 (d, *J* = 7.0)
	NH		7.52 (d, *J* = 10.0)
Pro	1"	171.8	
	2"	57.8	4.65 (dd, *J* = 2.0, 8.0)
	3"	24.7	1.76 (m), 2.32 (m)
	4"	25.0	2.17 (2H, m)
	5"	47.0	3.22 (m), 3.84 (m)
+Aoe	1"'	174.2	
	2"'	54.4	4.17 (dt, *J* = 10.0, 8.0)
	3"'	28.7	1.30 (2H, m)
	4"'	25.3	1.29 (2H, m)
	5"'	22.8	1.55 (2H, m)
	6"'	28.8	1.58 (m), 1.77 (m)
	7"'	36.3	2.25 (m), 2.38 (m)
	8"'	207.5	
	9"'	53.4	3.40 (dd, *J* = 4.0, 2.0)
	10"'	46.1	2.84 (dd, *J* = 6.0, 2.0), 2.96 (dd, *J* = 6.0, 4.0)
	NH		7.06 (d, *J* = 10.0)

^{13}C and ^1H NMR spectra were recorded at 125 MHz and 500 MHz, respectively.

+Iva: isovaline, Aoe: 2-amino-8-oxo-9,10-epoxydecanoic acid

The structure of **1** consists of four peptidyl moieties as follows. A ^{1}H-^{1}H spin coupling between a methyl proton 4-H and a methylene proton 3-H was observed in the COSY spectrum of **1**. The methylene proton and a singlet methyl proton 5-H displayed long-range couplings to a quaternary carbon C-2 and an amide carbonyl carbon C-1 in the HMBC spectrum. In addition to these correlations, long-range couplings from an amide proton at 5.77 ppm to C-2, C-3 and C-5 revealed the presence of an isovalyl residue (Fig. 2).

The COSY spectrum revealed the presence of a monosubstituted benzene residue and a sequence from an amide proton at 7.52 ppm to benzylic methylene protons 3′-H through an α-methine proton 2′-H as shown in Fig. 2. An amide carbonyl carbon C-1′ exhibited long-range couplings to 2′-H and 3′-H, which were in turn coupled to aromatic carbons C-4′ and C-5′, respectively. These data established the presence of a phenylalanyl residue.

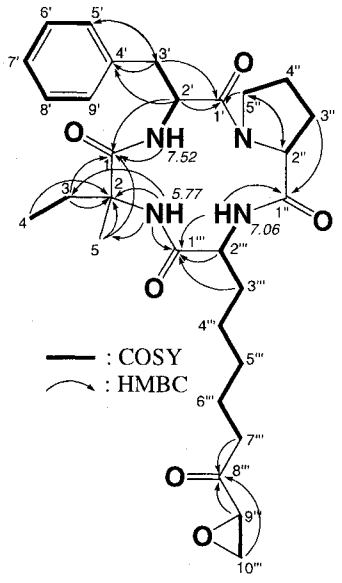
A proton spin system from 2″-H to 5″-H through 3″-H and 4″-H was detected in the COSY spectrum. C-2″ and C-5″ showed long-range correlations to their appended protons 5″-H and 2″-H, respectively. Moreover, 3″-H was coupled to an amide carbonyl carbon C-1″. These correlations as well as the chemical shifts of C-2″ (57.8 ppm) and C-5″ (47.0 ppm) proved the presence of a prolyl residue (Fig. 2).

The remaining amide proton at 7.06 ppm exhibited a sequence to 7‴-H, which was in turn long-range coupled to a carbonyl carbon C-8‴, through 2‴-H, 3‴-H, 4‴-H, 5‴-H and 6‴-H. A proton spin coupling between a methylene proton 10‴-H and a methine proton 9‴-H, which were long-range coupled to the ketone carbonyl C-8‴. A long-range coupling between 2‴-H and an amide carbon C-1‴ composed an amino acid residue.

The sequence of amino acid residues was established by long-range couplings from 2′-NH to C-1, from 5″-H to C-1′, from 2‴-NH to C-1″, and from 2-NH to C-1‴, respectively. By taking into consideration the molecular formula of **1** together with the high field ^{13}C chemical shifts of C-9‴ (53.4 ppm) and C-10‴ (46.1 ppm), these carbons were deduced to consist of an epoxide moiety, revealing a 2-amino-8-oxo-9,10-epoxydecanoic acid residue. Thus, the planar structure of **1** was determined to be a cyclic tetrapeptide as shown in Fig. 2. Phoenistatin (**1**) is structurally similar to chlamydocin[10], of which α-aminoisobutyryl was replaced by isovalyl residue. The determination of the absolute stereochemistry of **1** is now under way.

In the evaluation system we employed, the treatment of Mv1Lu cells with 40 ng/ml of TGF-β increased the expression of luciferase three-fold. **1** induced gene expression from the PAI-1 promoter more than three times at concentrations from 3 nM to 1000 μM. Since the structurally related compound chlamydocin had been reported to show cytostatic activity in mastocytoma cells[10], we next evaluated the effect of **1** on the cell growth and cytotoxicity by measuring MTT reduction and LDH release, respectively. **1** inhibited the growth of Mv1Lu cells with an IC_{50} of 1 μM as determined by the MTT assay. No LDH release was observed at the concentration showing the growth inhibitory effect, suggesting that **1** exhibited cytostatic activity without cytotoxicity in Mv1Lu cells.

Fig. 2. NMR analyses of **1**.



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References

1) MOSES, H. L.; E. L. BRANUM, J. A. PROPER & R. A. ROBINSON: Transforming growth factor production by chemically transformed cells. Cancer Res. 41:

2842~2848, 1981

2) MOSES, H. L.; E. Y. YANG & J. A. PIETENPOL: TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights. Cell 63: 245~247, 1990

3) MACKAY, S. L.; T. AUFFENBERG, C. L. TANNAHILL, R. KSONTINI, M. D. JOSEPHS, M. NOWAK, L. L. MOLDAWER & E. M. COPELAND: Transfection of the type II TGF-beta receptor into colon cancer cells increases receptor expression, inhibits cell growth, and reduces the malignant phenotype. Ann. Surg. 227: 781~789, 1998

4) GUO, Y. & N. KYPRIANOU: Restoration of transforming growth factor beta signaling pathway in human prostate cancer cells suppresses tumorigenictity *via* induction of caspase-1-mediated apoptosis. Cancer Res. 59: 1366~1371, 1999

5) LAIHO, M.; O. SAKSELA & J. KESKI-OJA: transforming growth factor-β induction of type-1 plasminogen activator inhibitor. Pericellular deposition and sensitivity to urokinase. J. Biol. Chem. 262: 17467~17414, 1987

6) ABE, M.; J. G. HARPEL, C. N. METZ, I. NUNES, D. J. LOSKUTOFF & D. B. RIFKIN: An assay for transforming growth factor-β using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. Anal. Biochem. 216: 276~284, 1994

7) TSUJI, N.; M. KOBAYASHI, K. NAGASHIMA, Y. WAKISAKA & K. KOIZUMI: A new antifungal antibiotic, trichostatin. J. Antibiotics 29: 1~6, 1975

8) YOSHIDA, M.; M. KIJIMA, M. AKITA & T. BEPPU: Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. J. Biol. Chem. 265: 17174~17179, 1990

9) MASUOKA, Y.; K. SHIN-YA, Y. -B. KIM, M. YOSHIDA, K. NAGAI, K. SUZUKI, Y. HAYAKAWA & H. SETO: Diheteropeptin, a new substance with TGF-β-like activity, produced by a fungus, *Diheterospora chlamydosporia*. I. Production, isolation and biological activities. J. Antibiotics 53: 788~792, 2000

10) CLOSSE, A. & R. HUGUENIN: Isolierung und strukturaufklärung von chlamydocin. Helv. Chim. Acta 57: 533~545, 1974