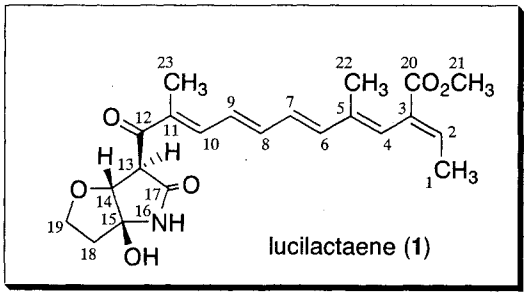
Lucilactaene, a New Cell Cycle Inhibitor in p53-Transfected Cancer Cells, Produced by a *Fusarium* sp.

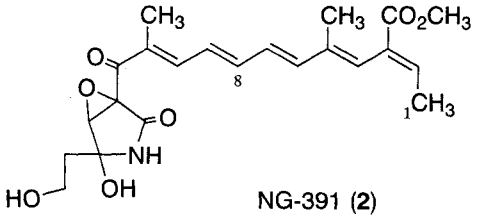
Sir:

The tumor-suppressor gene *p53* is involved in cell cycle control, apoptosis, differentiation, DNA repair and recombination, and other physiological events in response to a variety of stress signals[1,2]. The *p53* gene is lost or mutated in most human tumors. Lack of functional p53 is accompanied with high rates of genomic instability, rapid tumor progression, resistance to anticancer therapy, and increased angiogenesis[3,4]. The small molecules that induce cell cycle arrest or apoptosis p53-independently or allow mutant p53 to maintain an active form might be good candidates for anticancer drugs of various types of cancers[5,6]. For the bioassay to obtain such new small molecules produced by microorganisms, H1299/tsp53 cells were used as an indicator cell line. The transfectant H1299/tsp53 stably expresses a temperature-sensitive (Ala138>Val) human p53 in H1299 cells (human non-small cell lung cancer cells) which adopts the transcriptionally inactive, mutant conformation at the non-permissive temperature of 37°C, but behaves as a wild-type at the permissive temperature of 32°C[7]. In these cells, p53 is transcriptionally active only when the incubation temperature is shifted from 37°C to 32°C. During an extensive screening program, we found that a fungal strain, RK97-94, produced a new cell cycle inhibitor, lucilactaene (**1**, Fig. 1), that arrested the cell cycle progression at the G1 phase at 37°C in H1299/tsp53 cells. Here, we report the production, isolation, structure determination of lucilactaene and its effect on cell cycle progression.

The fungal strain RK97-94, to be identified as a *Fusarium* sp., was isolated from the leaf of an unidentified plant collected at Mt. Inasa, Nagasaki Prefecture, Japan, and deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-18143. For the production of **1**, the strain was inoculated into a seed medium consisting of 2% glucose, 1% soluble starch, 0.3% meat extract, 2.5% yeast extract, 0.05% NaCl, 0.005% K_2HPO_4, 0.05% $CaCO_3$, and 0.05% $MgSO_4 \cdot 7H_2O$ (adjusted at pH 7.2 before sterilization), and cultured on a rotary shaker (150 rpm) at 28°C for 48 hours. The seed culture (140 ml) was transferred into a 30-liter jar fermenter containing 15 liters of the same medium with antifoams. The fermentation was carried out at 28°C for 96 hours under constant agitation at 250 rpm and aerated 10 liters per minute.

Fig. 1. Structures of lucilactaene (**1**) and NG-391 (**2**).





The mycelia obtained from the whole culture broth (30 liters) were extracted with acetone, which was concentrated *in vacuo* to remove acetone. The resulting aqueous solution was extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to give an oily residue. This material was subjected to silica gel column chromatography with 0~50% methanol in chloroform stepwise. Lucilactaene (**1**) was eluted with 5% methanol in chloroform. Further purification was achieved by HPLC using a reverse phase column (PEGASIL ODS, 20 i.d.×250 mm, Senshu Scientific Co. Ltd, Tokyo; flow rate 9.0 ml/minute) eluted isocratically with 70% aqueous methanol. A pure sample of **1** (50.0 mg) was finally obtained as a pale yellow amorphous solid. The physico-chemical properties of **1** are summarized in Table 1. The molecular formula was established as $C_{22}H_{27}NO_6$ by high-resolution FAB-MS. IR absorptions at 3420, 1710, 1650 cm^{-1} implied the presence of a hydroxyl, a ketone, an ester, and an amide carbonyl functions. The UV spectrum had absorption maxima at 273 and 363 nm in methanol, suggesting the presence of a pentaene moiety linked to a ketone function. The ^{13}C and ^{1}H NMR spectral data in $CDCl_3$ are shown in Table 2.

Eight degrees of unsaturation could be accounted for by the presence of three carbonyl signals (a ketone resonance

Table 1. Physico-chemical properties of lucilactaene (**1**).

Appearance	Pale yellow amorphous solid
Molecular formula	$C_{22}H_{27}NO_6$
HRFAB-MS (m/z)	
Found	402.1923 $(M+H)^+$
Calcd	402.1917
UV λ_{max} nm (ε in MeOH)	273 (8800), 363 (48200)
IR ν_{max} (neat)	3420, 1710, 1650, 1580, 1435, 1255, 1135
Rf [a]	0.55 (chloroform-methanol=10 : 1)
Color reaction	10% H_2SO_4 (heat), Iodine

[a] Silica gel TLC (Merck $60F_{254}$)

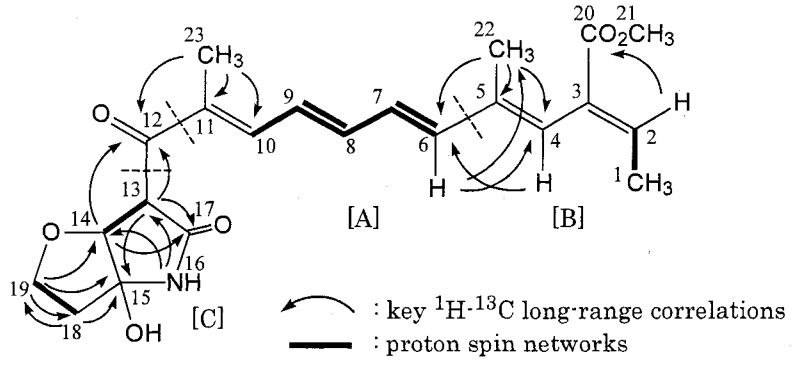
Table 2. ^{13}C and ^1H NMR data of lucilactaene (**1**) in $CDCl_3$.

Position	^{13}C [a]	^1H (multiplicity) [b]	J value (Hz)
1	15.94	1.75 dd	7.3, 1.4
2	140.56	7.01 qd	7.3, 0.9
3	130.32		
4	128.12	6.24 s	
5	137.98		
6	142.33	6.64 d	15.1
7	128.33	6.47 dd	15.1, 11.0
8	143.65	6.85 dd	14.7, 11.0
9	128.03	6.68 dd	14.7, 11.5
10	145.56	7.48 d	11.5
11	134.16		
12	196.99		
13	56.60	4.36 br s	
14	85.69	4.25 d [c]	0.9
15	94.44		
17	170.49		
18	37.45	2.28 ddd	12.8, 6.5, 3.8
		2.43 ddd	12.8, 8.8, 8.6
19	68.53	4.03 ddd	8.8, 8.8, 6.5
		4.13 ddd	8.8, 8.6, 3.8
20	167.45		
21	51.95	3.75 s	
22	14.26	1.72 d	1.4
23	11.55	1.96 s	

[a, b] ^{13}C and ^1H NMR spectra were recorded at 125 MHz and 500MHz, respectively. Chemical shifts in ppm are referred to TMS as an internal standard.

[c] Doublet due to coupling to the N-16 proton.

Fig. 2. NMR analyses of lucilactaene (**1**).

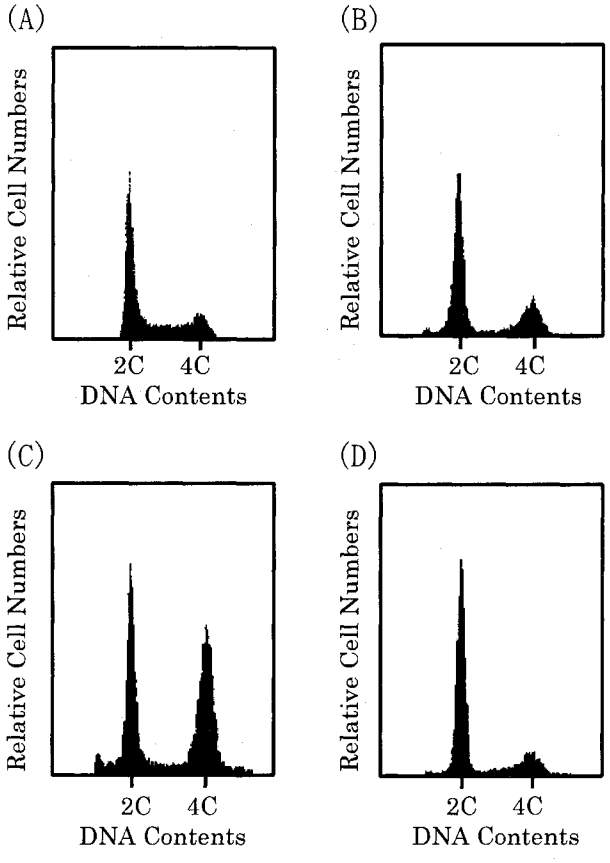


at 196.99 ppm, an amide at 170.49 ppm, and an ester at 167.45 ppm) and ten sp^2 carbon signals of five olefinic bonds. Therefore, the two remaining degrees of unsaturation indicated the presence of two rings. Detailed ^{1}H and ^{13}C NMR studies revealed the presence of three partial structures as shown in Fig. 2. A proton spin system from 6-H to 10-H though 7-H, 8-H, and 9-H was observed in the COSY spectrum. Long-range couplings from a methyl proton 23-H to C-10 and C-11 were observed in the HMBC spectrum, revealing the partial structure [A]. The ^{1}H–^{1}H spin coupling between methyl protons 1-H and 2-H was observed in the COSY spectrum. In addition, a methyl proton 22-H at 1.72 ppm displayed long-range couplings to a quaternary carbon C-5 and a sp^2 carbon C-4 in the HMBC spectrum, determining the partial structure [B]. Proton network systems between not only 13-H and 14-H but also 18-H and 19-H were detected in the COSY spectrum. Moreover, the following key long-range couplings established the presence of partial structure [C], a hexahydro-3a-hydroxy-5-oxo-2H-furo[3,2-b]pyrrol-6-yl ring system: H-13/C-15 and C-17; H-14/C-17; 16-NH/C-13 and C-14; H-19/C-14, C-15, and C-18; H-18/C-15 and C-19. The sequence of these partial structures and a remaining carbonyl carbon C-12 at 196.99 ppm was confirmed by the long-range couplings from H-13, H-14, and H-23 to C-12, from H-22 to C-6, from H-4 to C-6, and from H-6 to C-4 as shown in Fig. 2. High-field carbon shifts for the three allylic methyls and vicinal large coupling constants (J_{6-7}=15.1 Hz and J_{8-9}=14.7 Hz, respectively) indicated all *E* configurations for five olefinic bonds, which were confirmed based on NOEs between 1-H and 4-H, 4-H and 6-H, 6-H and 8-H, 8-H and 10-H, 7-H and 22-H, and 9-H and 23-H. Thus, the planar structure of **1** was determined as shown in Fig. 2. The relative stereochemistry of **1** was established as shown in Fig. 1 based on the following significant NOEs: 13-H/10-H; 14-H/15-OH and 16-NH; 16-H/15-OH and 18-H, which were in turn confirmed to be 13*S**, 14*R**, 15*S**.

We also isolated NG-391 (**2**, Fig. 1) and NG-393, a *Z* stereoisomer of **2** at the C-8 position, from the fermentation broth of *Fusarium* sp. RK97-94, which were previously reported as the neuronal cell-protecting molecules[8]. The structure similarity of **1** and **2** suggests that **2** might be a precursor of **1** as a result of the nucleophilic opening of the epoxy ring by the intramolecular hydroxy group of **2**. Epolactaene, which we have also reported as a neuritogenic molecule, possesses the same skeleton with an epoxide fused to a γ-lactam and a similar hydrophobic side chain to that of **1** and **2**[9~11]. Lucilactaene (**1**) is also structurally similar to fusarin A, a non-mutagenic metabolite of *Fusarium moniliforme*[12,13]. Study of the structure-activity relationships of these small molecules on our bioassay systems is now underway.

In H1299/tsp53 cells with a mutation in the *p53* gene, normal growth is observed at the non-permissive temperature of 37°C (Fig. 3-A), but the cells arrest at the G1 and G2 phase at the permissive temperature of 32°C (Fig. 3-B) as a result of activation of p53. As shown in Fig. 3-D, 5 μg/ml of **1** significantly augmentated 2C of the DNA contents after a 24 hours-incubation at 37°C, suggesting that **1** inhibited the cell cycle progression of H1299/tsp53 at the G1 phase at 37°C. Trichostatin A, a histone deacetylase inhibitor[14], arrested at both the G1 and G2 phases (Fig. 3-C). In addition, **1** also significantly activated the $p21^{WAF1}$ promoter, a target of the *p53* gene, in H1299/tsp53-luc cells[15], which was established by transfecting H1299/tsp53

Fig. 3. Lucilactaene (**1**) arrests the cell cycle progression at the G1 phase in H1299/tsp53 cells at the non-permissive temperature of 37°C.



The cells were maintained in 5% CO_2 humidified atmosphere in RPMI1640 containing 10% fetal bovine serum and 200 μg/ml of G418 on plastic culture dishes at the non-permissive temperature of 37°C. The cells were treated with 1% of methanol (A), 0.1 μg/ml of trichostatin A (C), or 5 μg/ml of lucilactaene (**1**) (D). After a 24-hours incubation at 37°C, the cells were harvested, and analyzed for DNA contents using a flow cytometer[19]. In (B), the DNA contents prepared from the cells cultured with 1% of methanol at the permissive temperature of 32°C for 24 hours were analyzed.

cells with a luciferase reporter gene linked at the 3′ of the human $p21^{WAF1}$ promoter[16~18]. Detailed studies on various kinds of cancer cell lines and the mode of action of **1** are also now being undertaken.

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References

1) LEVINE, A. J.: p53, The cellular gatekeeper for growth and division. Cell 88: 323~331, 1997
2) HANAHAN, D. & R. A. WEINBERG: The hallmarks of cancer. Cell 100: 57~70, 2000
3) LENGAUER, C.; K. W. KINZLER & B. VOGELSTEIN: Genetic instabilities in human cancers. Nature 396: 643~649, 1998
4) DAMERON, K. M.; O. V. VOLPERT, M. A. TAINSKY & N. BOUCK: Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science 265: 1582~1584, 1994
5) FOSTER, B. A.; H. A. COFFEY, M. J. MORIN & F. RASTINEJAD: Pharmacological rescue of mutant p53 conformation and function. Science 286: 2507~2510, 1999
6) VOGELSTEIN, B.; D. LANE, & A. J. LEVINE: Surfing the p53 network. Nature 408: 307~310, 2000
7) WINTERS, Z. E.; W. M. ONGKEKO, A. L. HARRIS & C. J. NORBURY: p53 Regulates Cdc2 independently of inhibitory phosphorylation to reinforce radiation-induced G2 arrest in human cells. Oncogene 17: 673~684, 1998
8) SUGAWARA, T.; H. TOKUNAGA & H. SIMURA: Japan Kokai Tokkyo Koho, 319289 (Dec. 3, 1996)
9) KAKEYA, H.; I. TAKAHASHI, G. OKADA, K. ISONO & H. OSADA: Epolactaene, a novel neuritogenic compound in human neuroblastoma cells produced by a marine fungus. J. Antibiotics 48: 733~735, 1995
10) KAKEYA, H.; C. ONOZAWA, M. SATO, K. ARAI & H. OSADA: Neuritogenic effect of epolactaene derivatives on human neuroblastoma cells which lack high-affinity

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nerve growth factor receptors. J. Med. Chem. 40: 391~394, 1997

11) HAYASHI, Y. & K. NARASAKA: Asymmetric total synthesis of epolactaene. Chem. Lett. 313~314, 1998

12) GELDERBLOM, W. C. A.; W. F. O. MARASAS, P. S. STEYN, P. G. THIEL, K. J. MERWE, P. H. ROOYEN, R. VLEGGAAR & P. L. WESSELS: Structure elucidation of fusarin C, a mutagen produced by *Fusarium moniliforme*. J. Chem. Soc., Chem. Commun. 122~125, 1984

13) STEYN, P. S. & R. VLEGGAAR: Biosynthetic studies on the fusarins, metabolites of *Fusarium moniliforme*. J. Chem. Soc., Chem. Commun. 1189~1191, 1985

14) FURUMAI, R.; Y. KOMATSU, N. NISHINO, S. KHOCHBIN, M. YOSHIDA & S. HORINOUCHI: Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin. Proc. Natl. Acad. Sci. USA 98: 87~92, 2001

15) NIE, L.; M. UEKI, H. KAKEYA & H. OSADA: A facile and effective screening method for $p21^{WAF1}$ promoter activators from microbial metabolites. J. Antibiotics 54: 783~788, 2001

16) EL-DEIRY, W. S.; T. TOKINO, V. E. VELCULESCU, D. B. LEVY, R. PARSONS, J. M. TRENT, D. LIN, W. E. MERCER, K. W. KINZLER & B. VOGELSTEIN: WAF1, a potential mediator of p53 tumor suppression. Cell 75: 817~825, 1993

17) HARPER, J. W.; G. R. ADAMI, N. WEI, K. KEYOMARSI & S. J. ELLEDGE: The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinase. Cell 75: 805~816, 1993

18) XIONG, Y.; G. J. HANNON, H. ZHANG, D. CASSO, R. KOBAYASHI & D. BEACH: p21 is a universal inhibitor of cyclin kinases. Nature 366: 701~704, 1993

19) KAKEYA, H.; R. ONOSE & H. OSADA: Inhibition of cyclin D1 expression and phosphorylation of retinoblastoma protein by phosmidosine, a nucleotide antibiotic. Cancer Res. 58: 704~710, 1998