COMMUNICATIONS TO THE EDITOR

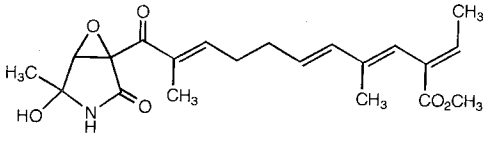
Epolactaene, a Novel Neuritogenic Compound in Human Neuroblastoma Cells, Produced by a Marine Fungus

Sir:

Neurotrophic factors are thought to be essential for survival and functional maintenance of nerve cells in the central and the peripheral nervous system[1,2]. The compounds which regulate the synthesis and secretion of neurotrophic factors might be a good candidate for pharmaceutical agents of various neurodegenerative diseases involving dementia. From this point of view, we started to explore neuritogenic compounds produced by microorganisms (mainly actinomycetes and fungi in the sea). For the bioassay to obtain the neuritogenic compounds, a human neuroblastoma cell line, SH-SY5Y cells[3] was used as an indicator. During the screening, we found that a fungal strain isolated from a sea sediment sample collected in the sea bottom of the Uchiura bay, Japan, produced a novel neuritogenic compound, epolactaene (Fig. 1). Here, we report production, isolation, structure determination and biological activities of epolactaene.

The strain identified to be *Penicillium* sp. BM1689-P was cultured in the seed medium composed of glucose 3.5%, soluble starch 1%, soybean meal 2%, polypeptone 0.5%, meat extract 0.3%, yeast extract 0.5%, NaCl 0.2%, KH_2PO_4 0.05% and $MgSO_4 \cdot 7H_2O$ 0.05% (adjusted at pH 5.8 before sterilization). The seed culture was carried out on a rotary shaker at 250 rpm for 48 hours in 500-ml cylindrical flasks containing 70 ml of the seed medium. Then, 140 ml of the culture was inoculated in a 30-liter jar fermenter containing 18 liters of the same medium with 0.05% of CA-123 and KM-68 antifoam. The fermentation was carried out at 27°C for 50 hours under constant agitation at 300 rpm and aerated 7 liters per minute. The purification procedure for epolactaene is outlined in Fig. 2. Epolactaene was extracted from mycelia by 80% acetone and purified by a successive column chromatography on silica gel (Silica gel 60, Merk) and Sephadex LH-20 (Pharmacia Fine Chemicals), and finally preparative HPLC (CAPCELL PAK C18, Shiseido). From 18 liters of the cultured broth, 10 mg of epolactaene was obtained as a colorless amorphous solid ($[\alpha]_D^{26}$ +32° (*c* 0.1, MeOH)) which gave a positive color response to iodine and H_2SO_4. The purified epolactaene gave two peaks on the HPLC analyses. Each peak was collected and analyzed by HPLC under the same conditions. Immediately after the separation, each fraction showed two peaks whose ratio was approximately 5 : 1 as a tautomeric mixture, which might be derived at the epimerization at C-15.

Fig. 1. Structure of epolactaene.



It is soluble in MeOH, EtOH and DMSO, slightly soluble in $CHCl_3$ and EtOAc, substantially insoluble in H_2O and *n*-hexane. The molecular formula of epolactaene was determined to be $C_{21}H_{27}NO_6$ from the result of HREI-MS spectrometry (Found: *m/z* 389.1847, Calcd: *m/z* 389.1846), which was supported by ^{13}C and ^{1}H NMR data (Table 1). The UV spectra showed absorption maxima at 232 nm (ε 21,800) and 280 nm (sh, ε 15,600) in MeOH. Its IR spectrum (KBr) revealed absorption at 3420, 2920, 1720, 1640, 1435, 1270, 1135, 970 and 965 cm^{-1}. The ^{13}C NMR spectrum exhibited signals of three carbonyl, eight olefinic carbons, two quaternary carbons, one oxygenated methine, two methylene, and five methyl carbons.

All the ^{1}H and ^{13}C signals were assigned as shown in Table 1. Detailed ^{1}H and ^{13}C NMR studies revealed the

Fig. 2. Purification procedure for epolactaene.

BM 1689-P
whole broth (18 liters)
↓ centrifuged
mycelial cake
↓ extracted 80 % acetone
concentrated *in vacuo*
adjusted to pH 7.0
extracted with EtOAc
organic layer
↓ concentrated *in vacuo*
crude compounds (4.7 g)
↓ Silica gel column
$CHCl_3$-MeOH stepwise (100:0, 100:1, 100:2, 100:5, 100:10, 100:20, 100:50, 0:100)
active fraction
↓ Sephadex LH-20 column
$CHCl_3$-MeOH (1:1)
active fraction
↓ prep. HPLC
CAPCELL PAK C18, 20 Φ x 250 mm (80% MeOH)
8.0 ml/min, 210 nm
active fraction
↓ prep. HPLC
CAPCELL PAK C18, 20 Φ x 250 mm (60% MeOH)
8.0 ml/min, 210 nm
epolactaene (10 mg)

This article is a special contribution in honour of Professor SATOSHI ŌMURA's 60th birthday.

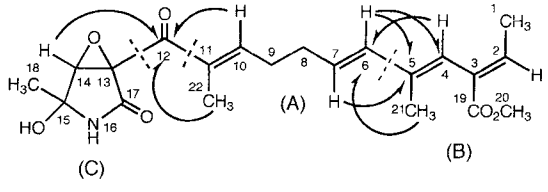
presence of three partial structures, (A), (B) and (C) as shown in Fig. 3. The cross peaks (6-H (δ 6.27)–7-H (δ 5.77)–8-H (δ 2.35)–9-H (δ 2.47)–10-H (δ 7.01)–22-H (δ 1.82)) observed in the ^{1}H-^{1}H COSY spectrum indicated the partial structure (A) of the unsaturated alkyl chain. The stereochemistry of the double bond (C-6, C-7) was determined as *E* configuration from the large coupling constant (J = 15.6 Hz).

Table 1. ^{13}C and ^{1}H assignments for epolactaene.

Position	^{13}C[a]	^{1}H (multiplicity)[a]	J value (Hz)
1	16.0	1.72 (dd)	1.2, 7.3
2	140.9	6.93 (dq)	1.0, 7.3
3	131.9		
4	123.7	5.94 (br s)	
5	139.6		
6	136.6	6.27 (d)	15.6
7	129.7	5.77 (dt)	15.6, 7.8
8	32.6	2.35 (m)	
9	30.1	2.47 (m)	
10	150.0	7.01 (dt)	1.0, 7.3
11	137.2		
12	192.1		
13	63.9		
14	66.1	3.98 (s)	
15	84.8		
17	172.2		
18	22.2	1.51 (s)	
19	169.6		
20	52.4	3.71 (s)	
21	14.6	1.61 (d)	1.3
22	11.1	1.82 (s)	

[a] 100 MHz (^{1}H) and 600 MHz (^{13}C) in CD_3OD. Chemical shifts in ppm from TMS as an internal standard.

Fig. 3. Partial structures (A, B, C) and connectivities among the partial structures by ^{1}H-^{1}H COSY and HMBC.



HMBC for the determination of the connectivities among the partial structures.

The cross peaks (1-H (δ 1.72)–2-H (δ 6.93) and 4-H (δ 5.94)–21-H (δ 1.61)) were also observed in the ^{1}H-^{1}H COSY spectrum. In addition, the heteronuclear multiple-bond correlation (HMBC) spectrum revealed the following two- and three-bonds connectivities between ^{1}H and ^{13}C: i) from 2-H to C-3 (δ 131.9), C-4 (δ 123.7) and C-19 (δ 169.6), ii) from 4-H to C-2 (δ 140.9), C-3 and C-19, iii) from 20-H (δ 3.71) to C-19. These spectral analyses indicated the partial structure (B).

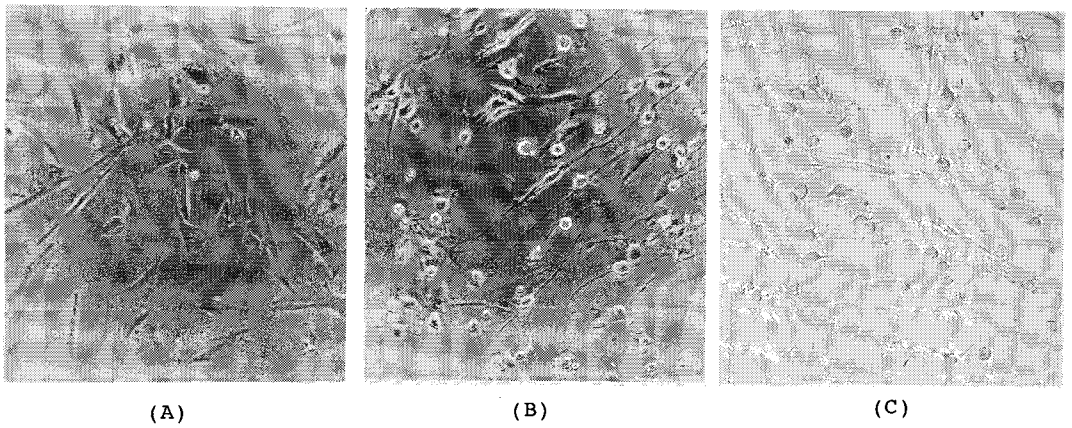
The partial structure (C) was also elucidated by the following connectivities in the HMBC spectrum: i) from 14-H (δ 3.98) to C-13 (δ 63.9), C-15 (δ 84.8) and C-18 (δ 22.2), ii) from 18-H (δ 1.51) to C-14 (δ 66.1) and C-15. Moreover, $^1J_{C14\text{-}H}$ coupling constant (197 Hz) suggested the presence of an epoxide ring at C-13 and C-14.

The connectivities of the three partial structures (A ~ C) were also elucidated by the HMBC spectrum (Fig. 3). The following cross peaks between ^{1}H and ^{13}C showed the connectivities between each partial structure: i) from 4-H and 21-H (δ 1.61) to C-6 (δ 136.6), while from 6-H and 7-H to C-5 (δ 139.6); (A) ~ (B), ii) from 10-H, 22-H and 14-H to the carbonyl C-12 (δ 192.1); (A) ~ (C).

The geometry of the three trisubstituted double bonds were determined by NOE difference spectra. NOEs observed between 1-H and 4-H, 4-H and 6-H, 7-H and 21-H, 9-H and 22-H established as 2*E*, 4*E*, and 10*E*.

Thus, the planner structure for epolactaene, containing an epoxide, γ-lactam, α,β-unsaturated ketone and triene moieties was determined as shown in Fig. 1. Epolactaene is a new type of neuritogenic compound. Fusarin C produced by *Fusarium moniliforme* is structurally related[4,5]. However, the reported biological activities of fusarin C was different from those of epolactaene.

Fig. 4. Morphological changes of SH-SY5Y cells treated with epolactaene or dibutyryl cAMP.



The cells were cultured at 37°C in 5% CO_2 humidified atmosphere in Basal Medium Eagle (BME) medium containing 10% fetal bovine serum on plastic culture dishes. Photograph were obtained after 2 days cultivation with/without epolactaene or dibutyryl cAMP. (A), control; (B), with 10 μg/ml of epolactaene; (C), with 1 mM of dibutyryl cAMP.

Epolactaene caused characteristic changes in the morphology of SH-SY5Y cells (Fig. 4). The control cells cultured without drugs extended quite a few neurites (<5% neurite-bearing cells/total cells in average). When the cells were treated with epolactaene at 2.5~10 μg/ml, many neurites were extended from cell bodies in a dose-dependent manner (74% neurite-bearing cells/total cells in average at 10 μg/ml). Neurite formation in SH-SY5Y cells was also induced by 1 mM of dibutyryl cAMP (87% neurite-bearing cells/total cells in average).

Recently, several compounds which induce neurite extension of mouse neuroblastoma, Neuro 2A cells have been reported, for example, lactacystin[6] and PS-990[7]. To our best knowledge, however, epolactaene is the first microbial metabolite effective to the neurite outgrowth of a human neuroblastoma cell line. Detailed studies on biological activities and the mechanism of action of epolactaene are being now undertaken.

Acknowledgements

We are grateful to Drs. H. MATSUI and M. INO (St. Marianna University) for the gift of SH-SY5Y cells. We would also like to thank Dr. H. KOSHINO (RIKEN) for the measurement of NMR spectra. A part of this work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

HIDEAKI KAKEYA
ISAMU TAKAHASHI†
GEN OKADA
KIYOSHI ISONO†
HIROYUKI OSADA

The Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351-01, Japan
†Kaken Pharmaceutical Co. Ltd., Gensuke, Fujieda, Shizuoka 426, Japan

(Received February 28, 1995)

References

1) VANTINI, G. & S. D. SKAPER: Neurotrophic factors; from physiology to pharmacology?. Pharmacological Research 26: 1~15, 1992
2) BARDE, Y.-A.: Trophic factors and neuronal survival. Neuron 2: 1525~1534, 1989
3) ROSS, R. A. & J. L. BIEDLER: Presence and regulation of tyrosinase activity in human neuroblastoma cell variants *in vitro*. Cancer Res. 45: 1628~1632, 1985
4) GELDERBLOM, W. C. A.; W. F. O. MARASAS, P. S. STEYN, P. G. THIEL, K. J. VAN DER MERWE, P. H. VAN ROOYEN, R. VLEGGAAR & P. L. WESSELS: Structure elucidation of fusarin C, a mutagen produced by *Fusarium moniliforme*. J. Chem. Soc., Chem. Commun. 1984: 122~124, 1984
5) GADDAMIDI, V.; L. F. BJELDANES & J. N. SHOOLERY: Fusarin C, structure determination by natural abundance ^{13}C-^{13}C coupling and deuterium-induced ^{13}C shifts. J. Agric. Food Chem. 33: 652~654, 1985
6) ŌMURA, S.; T. FUJIMOTO, K. OTOGURO, K. MATSUZAKI, R. MORIGUCHI, H. TANAKA & Y. SASAKI: Lactacystin, a novel microbial metabolite, induces neuritogenesis of neuroblastoma cells. J. Antibiotics 44: 113~116, 1991
7) TOKI, S.; K. ANDO, M. YOSHIDA & Y. MATSUDA: PS-990, a novel neuritogenic compound from *Acremonium* sp. J. Antibiotics 47: 1175~1181, 1994