COMMUNICATIONS TO THE EDITOR

Acetophthalidin, a Novel Inhibitor of Mammalian Cell Cycle, Produced by a Fungus Isolated from a Sea Sediment

Sir:

In the course of our screening for new inhibitors of mammalian cell cycle[1], we have previously reported two new compounds, tryprostatins A and B, isolated from the culture broth of *Aspergillus fumigatus*, which inhibit the mammalian cell cycle in the G2/M phase[2]. In the continued screening, we have now discovered a novel inhibitor named acetophthalidin (**1**) (Fig. 1). Acetophthalidin was produced by a fungal strain BM923 isolated from a sea sediment, however, it was hardly possible to isolate **1** directly from the culture broth of the producing strain because **1** was readily degraded into inactive form(s) during the separation. As we have proved that **1** was converted from 3,4,6-trihydroxymelleine (**2**) by the boiling in an acidic condition (Fig. 1), **2** was purified as a proto-inhibitor from the fermentation of the fungal strain BM923.

The producing strain was isolated from a sea sediment sample collected in the sea bottom of the off shore from Miho, Sizuoka prefecture, Japan, and was identified as a fungus belonging to the genus *Penicillium* through a taxonomic study. The species was not determined yet. In this communication, we preliminarily report the discovery, structure and biological activity of **1**.

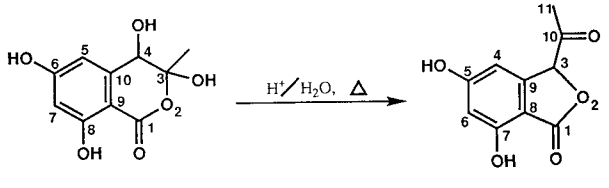
The biological examination for this work was carried out according to the previously reported method[2] by the use of a mouse tsFT210 cell line which is a temperature-sensitive $p34^{cdc2}$ mutant. The cells were growing normally at 32°C, but arrested in the G2 phase at 39°C[2~4].

In a preliminary examination, we found that the fresh culture broth of the fungal strain BM923 strongly inhibited the cell cycle progression of mouse tsFT210 cells, but the activity disappeared during the purification. In a followed stability test, however, the inhibitory activity which has been lost once during the experiments reappeared after a treatment of the sample, just before the biological examination, by heating a water solution of the sample at pH 1 for 20 minutes in a boiling-water bath. This result was constantly observed in every tests repeated for the confirmation. Thus, the following separation procedure was monitored by the inhibitory activity observed after the treatment of the sample in the same manner. This research procedure resulted in the isolation of a proto-inhibitor (**2**) as described bellow.

Spores of the producing strain was inoculated into each of two 500-ml Erlenmeyer flasks containing 70 ml of a medium consisting of glucose 3%, soluble starch 2%, soybean meal 2%, K_2HPO_4 0.5% and $MgSO_4 \cdot 7H_2O$ 0.05% (pH 6.5) and cultured at 28°C for 48 hours on a rotary shaker at 300 rpm. The seed culture (3.5 ml) was then inoculated in each of thirty eight 500-ml Erlenmeyer flasks containing 70 ml of the same medium and cultured for 84 hours under the same condition. The isolation procedure for **2** is outlined in Fig. 2. The cultured whole broth was centrifuged to obtain a mycelial cake, which was extracted with 80% aqueous acetone. The aqueous acetone solution was evaporated *in vacuo* to remove acetone and then extracted with the same volume of EtOAc to give an extract (5 g) from which, **2** was isolated by HPLC (CAPCELL PAK C_{18}, Shiseido) separation followed by the recrystalization from 80% MeOH (Fig. 2). From 2.5 liters of the cultured broth, 966 mg of **2** was obtained as colorless prisms.

The proto-inhibitor, **2**, was then converted entirely into a genuine inhibitor, **1**, by heating a water solution

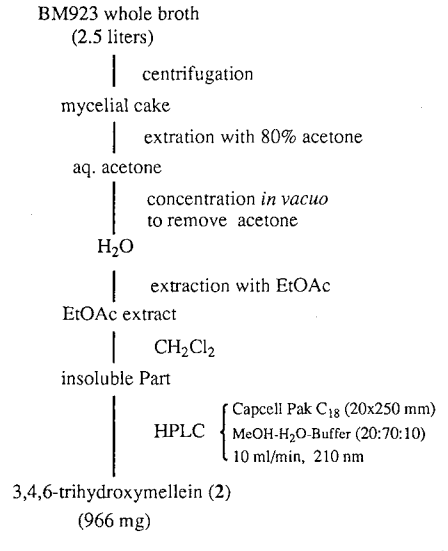
Fig. 1. Structures of acetophthalidin (**1**) and 3,4,6-trihydroxymelleine (**2**).



2 **1**

2 was easily converted to **1** by the boiling in an acidic condition.

Fig. 2. Purification procedure for 3,4,6-trihydroxymelleine (**2**).



Preparation of the buffer for HPLC: Formic acid was added to an aqueous solution containing 1% of the diethylamine until the solution shows pH 3.

(10 mg/ml; 1 ml; pH 1, adjusted with 1 N HCl) of **2** for 20 minutes in a boiling-water bath (Fig. 1). Pure **1** was obtained as a crystalline powder, without any purification, through the rapid removal of water immediately by the use of a nitrogen gas current.

Both **1** and **2** were obtained as an optically inactive form. The physico-chemical properties of **1** and **2** are summarized in Table 1. The molecular formulae, $C_{10}H_8O_5$ for **1** and $C_{10}H_{10}O_6$ for **2**, were determined by positive and negative FAB-MS measurements and elemental analyses (Table 1). The resemblance of UV absorption of **1** and **2** (Table 1) reveals the presence of the same chromophore in both **1** and **2**, and the IR spectrum of **1** showed an additional absorption at 1720 cm^{-1} in comparison with that of **2**, as shown in Table 1, indicating the presence of an additional ketocarbonyl group in 1. The ^1H and ^{13}C NMR spectra of **1** and **2** were respectively given in Figs. **3** and **4**. In the NMR spectra, **2** showed two sets of signals assigned to a pair of isomers, while **1** showed only one set. The presence of a ketocarbonyl group in **1** was also supported by the ^{13}C signal at 203.3 ppm (C-10) in the ^{13}C NMR spectrum of **1** (Fig. 4). Eventually, **1** was determined to be a new compound with a 5,7-dihydroxyphthalide skeleton and **2** was identified as a known 3,4,6-trihydroxymelleine[5], as shown in Fig. 1, by detailed analyses of their ^1H and ^{13}C NMR spectra with the aid of 2D NMR techniques including pulse field-gradient heteronuclear multiple-bond correlation (PFG-HMBC) spectroscopy. From a consideration of the optically inactive properties of **1** and **2**, coupled with their NMR spectral observations, **1** should be a racemate and **2** be a mixture of *cis*, *trans* isomers each of which respectively consists of two enantiomers.

Then, we prepared rapidly a fresh EtOAc-extract, under a mild condition, from a newly cultured whole broth of the producing strain BM923 under the same culture condition as mentioned above. The extract was immediately subjected to a HPLC analysis on a Senshu PAK C_6H_5-1252N column (4.6 × 250 mm, Senshu Sciences Co., Ltd., Japan) using MeOH-H_2O (60:40) as an eluting solvent by the use of a HPLC equipment system with a Waters 991J photodiode array detector. Both **1** and **2** were detected from the fresh extract with

Table 1. Physico-chemical properties of **1** and **2**.

Characteristics	1	2
Appearance	White crystalline powder	Colorless prisms
MP	195-205°C (dec.)	204-206°C
$[\alpha]_D^{24}$ (MeOH)	0° (c 0.1)	0° (c 1.0)
Molecular Formular	$C_{10}H_8O_5$	$C_{10}H_{10}O_6$
Molecular Weight	208	226
FAB-MS *m/z*		
Positive	209 $[M+H]^+$	227 $[M+H]^+$
Negative	207 $[M-H]^-$	225 $[M-H]^-$
Elementary analysis		
Found	C 56.06, H 3.78	C 53.07, H 4.42
Calcd for	$C_{10}H_8O_5 \cdot 1/3H_2O$	$C_{10}H_{10}O_6$
	C 56.08, H 4.08	C 53.10, H 4.45
UV λ_{max}^{MeOH} nm (ε)	223 (14130)	216 (13240)
	225 (sh, 13680)	224 (sh, 11130)
	258 (10580)	264 (6780)
	294 (4305)	301 (4620)
IR ν_{max}^{KBr} cm^{-1}	3310, 3160 br,	3400, 3315, 3120 br,
	1720, 1635, 1618,	1635, 1620,
	1480, 1365, 1160,	1467, 1320, 1165,
	1023, 845, 760	1060, 845, 780

Fig. 3. ^1H NMR spectra of acetophthalidin (**1**) and 3,4,6-trihydroxymelleine (**2**) in DMSO-d_6 (500 MHz)

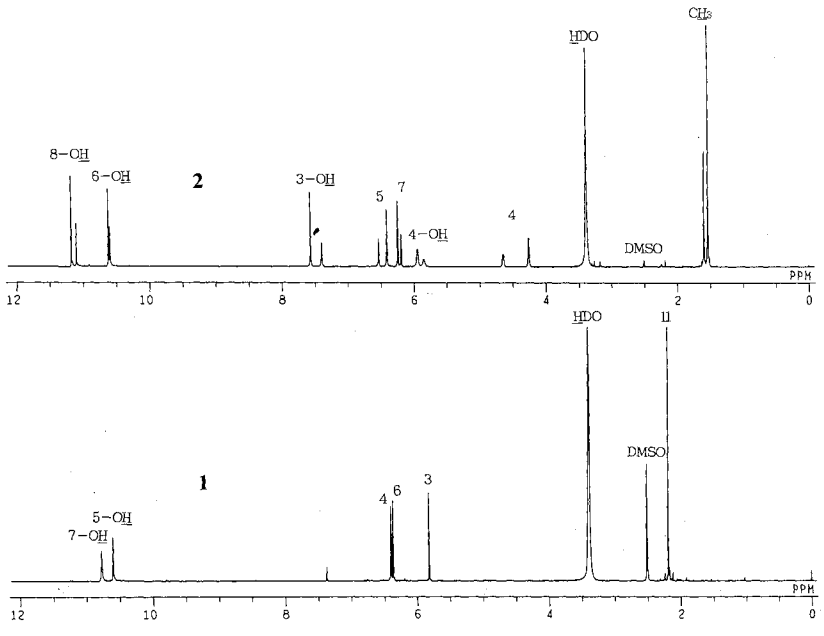


Fig. 4. ^{13}C NMR spectra of acetophthalidin (**1**) and 3,4,6-trihydroxymelleine (**2**) in DMSO-d_6 (125 MHz)

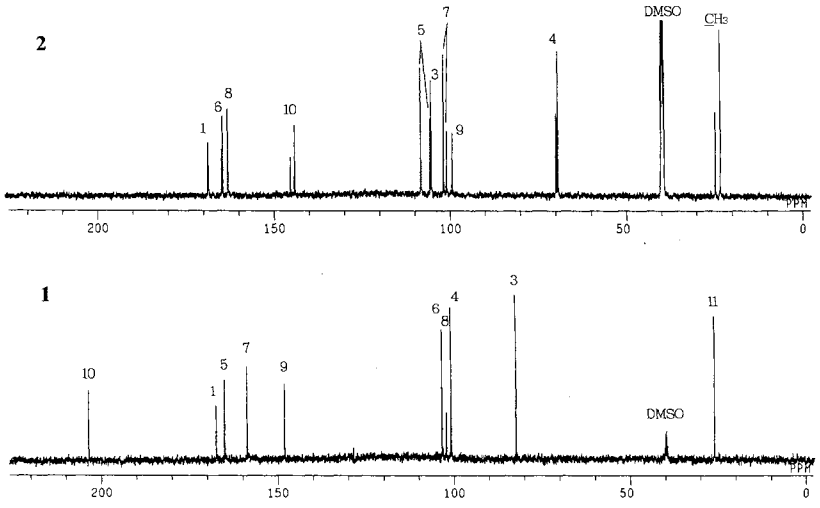
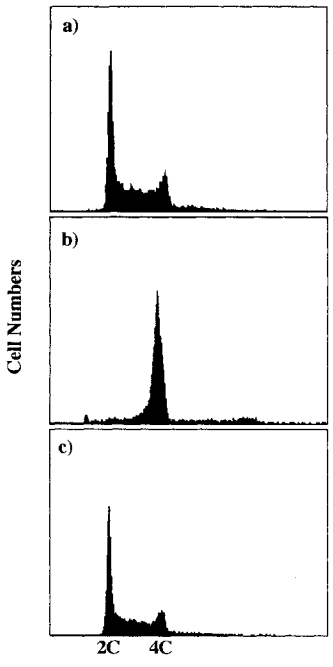


Fig. 5. Effects of **1** and **2** on the cell cycle progression of tsFT210 cells.



The tsFT210 cells were cultured at 32°C for 17 hours at a density of 2×10^5 cells/ml in RPMI-1640 medium supplemented with 5% calf serum: a) no drug control, b) acetophthalidin (**1**, 6.25 μg/ml), c) 3,4,6-trihydroxymelleine (**2**, 100 μg/ml).

the retention times, 3.9 minutes for **1** and 3.0 minutes for **2**, respectively, suggesting that **1** is also a natural product produced by the fungal strain BM923.

Acetophthalidin (**1**) completely inhibited the cell cycle progression of tsFT210 cells in the M phase at a final concentration of 6.25 μg/ml, while **2** showed no any effect even at 100 μg/ml (Fig. 5). Further biological studies of **1** are currently undertaken.

Even though many phthalides had so far been known in the nature[6~8], the present result provides acetophthalidin (**1**), a new phthalide carrying an acetonyl group at the C-3 position, as a novel inhibitor of mammalian cell cycle, which is unstable especially in the aqueous solution probably because of the presence of the acetonyl group at the C-3 position. On the other hand, the present work shows an unique research approach for the discovery of a new, biologically active and unstable or convertible compound from the microbial metabolites.

Details of the studies will be reported in a forthcoming paper.

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