Diazaphilonic Acid, a New Azaphilone with Telomerase Inhibitory Activity

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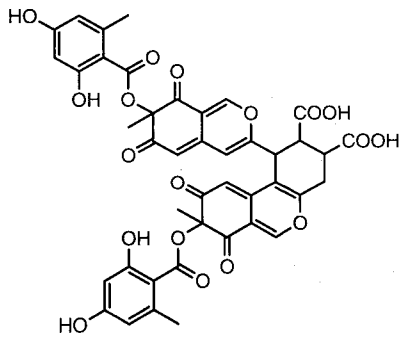
Through our screening program of bioactive secondary metabolites of microbial origin, we have isolated a new azaphilone-type metabolite, diazaphilonic acid (Fig. 1). In this paper, we report the taxonomy of the producing strain, production, isolation, physico-chemical properties and biological activities of diazaphilonic acid.

The mycological characteristics of the producing fungal strain PF1195 (Fig. 2) were as follows. Yellow colonies grew rapidly at 25°C on Czapek-yeast extract agar and malt extract agar. Ascomata were abundantly produced and conidiogenesis was sparse and inconspicuous. Ascomata were non-ostiolate, yellow, formed a telaperidium, and matured within 14 days. Ascomatal initials consisted of clavate cells, around which thin hyphae were coiled tightly several times. Asci were borne in chains and were 8-spored. Ascospores were ellipsoidal, $3.5\sim4.5\times2\sim3\,\mu m$ and spinulose. Penicilli were bivert-icillate. Conidia were subglobose to ellipsoidal, $2\sim3\times1.5\sim2\,\mu m$ and smooth walled. The growth-rate at 37°C was more rapid than at 25°C. Based on the properties above, strain PF1195 was identified as *Talaromyces flavus*.[1)]

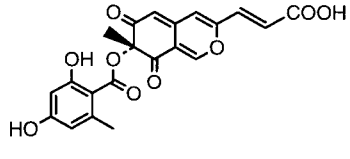
Diazaphilonic acid was produced as follows by fermentation of *Talaromyces flavus* PF1195. Strain PF1195, grown on an agar slant, was inoculated into a 100-ml Erlenmeyer flask that contained 20 ml of a seed medium consisting of 1.0% starch, 1.0% glucose, 0.6% wheat germ, 0.2% soybean meal, 0.3% yeast extract, 0.5% peptone, 0.2% $CaCO_3$ and tap water (pH 7.0 before sterilization). The inoculated flask was shaken on a rotary shaker (200 rpm) at 25°C for 2 days. This seed culture (5 ml) was added to 500-ml Erlenmeyer flasks that contained 100 g of a production medium consisting of soaked rice and 2.5% of soybean meal. The inoculated flasks were incubated as stationary cultures for 14 days at 28°C.

Isolation of diazaphilonic acid was performed as follows. Aqueous 67% acetone (2.5 liters) was added to the production medium (1 kg) of *Talaromyces flavus* PF1195 and the mixture was agitated for 1 hour. The extract was concentrated to remove the acetone and the remaining aqueous solution was adjusted to pH 3.2 with HCl (6 N). Then the solution was extracted with ethyl acetate (1 liter). The organic layer was evaporated *in*

Fig. 1. Structure of diazaphilonic acid (**1**) and its related compound, mitorubrinic acid (**2**).



Diazaphilonic acid (**1**)



Mitorubrinic acid (**2**)

Fig. 2. Ascospores of *Talaromyces flavus* PF1195.

Scale bar represents 5 μm.

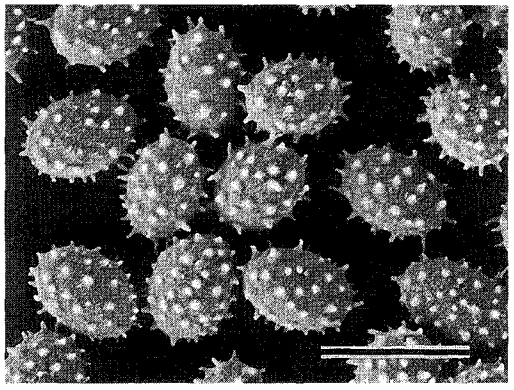
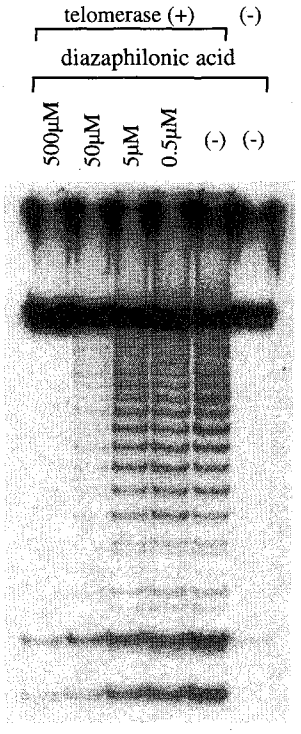


Table 1. Physico-chemical properties of diazaphilonic acid.

Appearance	Yellow powder
Molecular formula	$C_{42}H_{32}O_{18}$
HRFAB-MS (*m/z*)	
Found	825.1661
Calcd.	825.1668 for $C_{42}H_{32}O_{18}$
$[\alpha]_D^{25}$ (*c* 0.5, MeOH)	−371
UV λ_{max}^{MeOH} nm (ε)	218 (50500), 269 (29600), 325 (35000)
IR_{max} cm^{-1} (KBr)	3400, 1720, 1624, 1595, 1543, 1450
Solubility (soluble)	DMSO, MeOH

Fig. 3. Inhibitory effects of diazaphilonic acid on telomerase activity.



vacuo to yield a brown powder (3.7 g).

An aliquot of acquired brown powder (2.0 g) was subjected to silica gel (Wako Gel C-300) column chromatography (65 × 40 mm) developed with $CHCl_3$-MeOH. The fraction developed with $CHCl_3$-MeOH (20 : 1) was evaporated *in vacuo* to give a brown powder (583 mg). The resulting powder was dissolved and applied in four portions to preparative HPLC (column, Shiseido CAPCELL PAK C_{18}, 20 mm × 250 mm, flow rate 10 ml/minute). The column was eluted with CH_3CN-0.1% aqueous trifluoroacetic acid (1 : 1) with UV detection at 254 nm. The yield of diazaphilonic acid (yellow powder) was 209 mg.

The physico-chemical properties of diazaphilonic acid are summarized in Table 1. Diazaphilonic acid is a dimer of mitorubric acid[2] (Fig. 1). NMR spectral data and structure determination will be described in a separate report[3].

Diazaphilonic acid inhibited DNA amplification by polymerase chain reaction (PCR) with Tth DNA polymerase. The IC_{50} value was 2.6 μg/ml.

Diazaphilonic acid also inhibited telomerase activity (Fig. 3). After incubation of the telomerase assay mixture with diazaphilonic acid, telomere DNA produced by telomerase was captured, washed to remove diazaphilonic acid, and amplified by PCR. Diazaphilonic acid dose-dependently inhibited telomerase activity of MT1 (human leukemia) and almost completely inhibited activity at 50 μM.

Antimicrobial activities of diazaphilonic acid were evaluated by the agar hole method (40 μl of 1 mg/ml solution). It showed no antimicrobial activity against Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC9341, *Staphylococcus aureus* 209P), Gram-negative bacteria (*Escherichia coli* NIHJ) or several kinds of yeast (*Saccharomyces cerevisiae* SHY3, *Candida albicans* M9001, *Candida pseudotropicalis* M9035, *Cryptococcus neoformans* M9010,

Debaryomyces hansenii M9011, *Trigonopsis variabilis* M9031, *Schizosaccharomyces pombe* M9025 and *Hansenula schneggi* IAM4269).

Experimental Procedures

PCR Inhibition Assay

The PCR inhibition assay was performed using a modified version of the method of SCHOENFELD *et al.*[4] The PCR assay mixture (total 70 μl) consisted of 18 mM Tris buffer (pH 8.3), 1.5 mM $MgCl_2$, 54 mM KCl, 0.0045% Tween20, 0.9 mM EGTA, dATP 36 μM, dCTP 36 μM, dGTP 36 μM, TTP 1.4 μM, ^{3}H-TTP (4.4 TBq/mmol, Amersham Pharmacia Biotech) 132 kBq/ml, BSA 0.07 mg/ml, template DNA, two primers 0.14 μg/ml with one being biotinylated, Tth DNA polymerase (Toyobo Co., Ltd.) 0.25 U and diazaphilonic acid solution. The reaction mixture was subjected to 26 cycles of 94°C for 30 seconds, 53°C for 60 seconds, and 72°C for 90 seconds with a DNA thermal cycler. 50 μl of the mixture was removed to an OptiPlate (Packard) and mixed with 20 μl of SPA beads suspension (7.5 mg/ml, Amersham Pharmacia Biotech) and 50 μl of water. The OptiPlate was sealed with Top Seal A (Packard) and incubated at room temperature overnight. The radioactivity of ^{3}H-TTP incorporated into amplified DNA was counted with a 96-well liquid scintillation counter. (Top count, Packard).

Telomerase Inhibition Assay

This assay was performed by the modified method of KIM *et al*[5]. The telomerase assay mixture consisted of 5 μl of assay buffer (200 mM Tris buffer (pH 8.3), 15 mM $MgCl_2$, 600 mM KCl, 0.05% Tween20, 10 mM EGTA), 20 μl of dNTP (0.125 mM), 2 μl of primer A (5'AATCCGTCGAGCAGAGTT3') (0.05 μg/ μl) , 1 μl of T4 gene 32 (0.5 mg/ml), 5 μl of BSA solution (1 mg/ml), 2 μl of cell extract of MT1 cells (leukemia, human) and 5 μl of diazaphilonic acid solution. The reaction mixture was incubated for 20 minutes at 23°C, followed by incubation for 2 minutes at 94°C to stop the reaction. A 3'-biotinylated primer A' (5'AACTCTGCTCGACGGATT3') solution (4 μl, 0.05 μg/ μl) was added to the reaction mixture followed by incubation for 5 minutes at 94°C. 10 μl of NaCl solution (5 M) was added to the reaction mixture followed by incubation for 15 minutes at 37°C. After incubation, 8 μl of streptavidin-coated magnet beads suspension (5 mg/ml) was added to the mixture followed by incubation for 15 minutes at room temperature. The magnet beads were captured by a magnet and the supernatant of the reaction mixture was removed. The captured beads were washed with TE buffer (10 mM Tris buffer (pH 8.0), 1 mM EDTA, 0.1 M NaCl) three times to wash out the test compound (diazaphilonic acid). 50 μl of distilled water was added to the beads and incubated at 70°C for 5 minutes. The supernatant (22 μl) was mixed with 5 μl of assay buffer, 5 μl of dNTP solution (0.5 mM), 1 μl of T4 gene 32 solution (0.5 mg/ml), 5 μl of BSA solution (1 mg/ml), 2 μl of primer A solution (0.05 μg/ μl), 2 μl of primer B (5'CCCTTACCCTTACCCTTACCCTAA3') solution (0.05 μg/ μl), 4 μl of α-^{32}P-dCTP solution (1 μCi/ μl, Amersham Pharmacia Biotech) and 4 μl of Taq DNA polymerase solution (0.5 U/ μl). The mixture was subjected to 30 cycles of 94°C for 30 seconds, 50°C for 60 seconds, and 72°C for 90 seconds. The PCR products were extracted with $CHCl_3$ and electrophoresed on 10% acrylamide gel. After electrophoresis, the products of telomerase were analyzed by an imaging analyzer (BAS-2000, Fujix).

Acknowledgments

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