Diazaphilonic Acid, a New Azaphilone with Telomerase Inhibitory Activity

YUJI TABATA, SHUJI IKEGAMI1, TAKASHI YAGUCHI, TORU SASAKI, SHIGERU HOSHIKO, SADATOSHI SAKUMA1, KAZUO SHIN-YA1 and HARUO SETO1

Drug Discovery, Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., 760 Morooka-cho, Kohoku-ku, Yokohama 222-8567, Japan
1Meiji Institute of Health Science, Meiji Milk Products Co., Ltd., 540 Naruda, Odawara 250-0862, Japan
11Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan

(Received for publication November 13, 1998)

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, 3.5~4.5 x 2~3 μm and spinulose. Penicilli were biverticillate. Conidia were subglobose to ellipsoidal, 2~3 x 1.5~2 μm 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% CaCO3 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).
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 x 40 mm) developed with CHCl₃-MeOH. The fraction developed with CHCl₃-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 C₁₈, 20 mm x 250 mm, flow rate 10 ml/minute). The column was eluted with CH₃CN-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 acid² (Fig. 1). NMR spectral data and structure determination will be described in a separate report³).

Diazaphilonic acid inhibited DNA amplification by polymerase chain reaction (PCR) with Tth DNA polymerase. The IC₅₀ 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, Candida tropicalis M9035, Cryptococcus neoformans M9010, Candida albicans M9001, Candida tropicalis M9035, Cryptococcus neoformans M9010, Candida albicans M9001, Candida tropicalis 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 MgCl₂, 54 mM KCl, 0.0045% Tween20, 0.9 mM EGTA, dATP 36 µM, dCTP 36 µM, dGTP 1.4 µM, 3H-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 3H-TTP incorporated into amplified DNA was counted with a 96-well liquid scintillation counter. (Top count, Packard).

Telomerase Inhibition Assay
This assay was performed using the modified version of Kim et al.3). The telomerase assay mixture consisted of 5 µl of assay buffer (200 mM Tris buffer (pH 8.3), 15 mM MgCl₂, 600 mM KCl, 0.05% Tween20, 10 mM EGTA), 20 µl of dNTP (0.125 mM), 2 µl of primer A (5’ AATCC-GTCGAGCGAGTT3’ ) (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 primer A solution (0.05 µg/µl), 2 µl of primer B (5’ CCGCTCCGAGCTCCGGTTA3’ ) solution (0.05 µg/µl), 4 µl of α-32P-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 CHCl₃ and electrophoresed on 10% acrylamide gel. After electrophoresis, the products of telomerase were analyzed by an imaging analyzer (BAS-2000, Fujix).

Acknowledgments
This work was supported in part by Grant-in-Aid for Cancer Research, The Ministry of Education, Science and Culture, Japan to H. S.

References