A NEW SESQUITERPENE ANTIBIOTIC, HEPTELIDIC ACID PRODUCING ORGANISMS, FERMENTATION, ISOLATION AND CHARACTERIZATION

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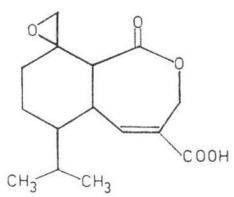
(Received for publication February 8, 1980)

A new sesquiterpene antibiotic, heptelidic acid, was found in the culture filtrate of three different strains of fungi isolated from soil samples. These strains were identified as *Gliocladium virens*, *Chaetomium globosum* and *Trichoderma viride*. Heptelidic acid was produced by conventional submerged culture and purified by successive column chromatography on silica gel and Sephadex LH-20 and finally by preparative TLC on silica gel. The molecular formula of heptelidic acid was determined as $C_{15}H_{20}O_5$ on the basis of elementary analysis and high resolution mass spectrometry of its monomethyl ester. The antimicrobial spectrum of the antibiotic revealed its specific activity against anaerobic bacteria, especially against *Bacteroides fragilis*.

In the course of our search for new antibiotics, an antibiotic primarily active against *Bacteroides fragilis* has been found in the culture filtrate of three different strains of fungi. These producers were identified as *Gliocladium virens*, *Chaetomium globosum* and *Trichoderma viride*, respectively, while the antibiotic was named heptelidic acid after the characteristic chemical functions in its structure.

The present paper deals with identification of the producing organisms, fermentation, isolation and physico-chemical and biological properties of heptelidic acid. Structural elucidation of heptelidic acid will be reported in the subsequent paper.

Chart 1.



Identification of the Producing Fungi

Strain SANK 12679 was freshly isolated from a soil sample collected at Sagamihara City, Kanagawa Prefecture, Japan. From the characters described below and by comparison with an authentic strain ATCC 9645, the fungus was identified as *Gliocladium virens*.

Gliocladium virens MILLER *et al.*, in Mycologia, 49: 792, 1975.[1]

Colonies on potato dextrose agar grow rapidly, attaining a diameter of 7~8 cm in 7 days at 26°C, plane; it is floccose with convoluted surface, dark green to olive green. Conidial structures are abundantly produced; reverse is uncolored.

Cultural characteristics on malt extract agar are nearly the same as those on potato dextrose agar.

At 37°C, growth is nil.

Conidiophores are erect or recumbent, single or in groups, septate and nearly smooth, 30~100×4~6 μ. Conidial apparatus are variously produced, composed of irregularly or truly penicillate. Penicilli are asymmetrical to symmetrical. Metulae are 2~4 in number, cylindrical, 10~20×2.5~3.0 μ.

Phialides are lageniform and various in size, commonly $3\sim6\times2\sim4\ \mu$. Conidia are mostly subglobose, sometimes ovoid to reniform, $2.5\sim4.5\times3.0\sim7.5\ \mu$, pale green, smooth-walled, aggregated into gelatinous balls or masses and green in color. Chlamydospores are not produced.

Strain SANK 13379 was freshly isolated from a soil sample collected in Indonesia. From the characters described below, the fungus was identified as *Chaetomium globosum*.

Chaetomium globosum KUNZE *ex* FRIES, in Syst. Mycol. 3: 225, 1829; AMES, A monograph of the Chaetomiaceae, p. 26, 1961.[2]

Cultures on WEITZMAN and SILVA-HUTNER's agar[3] spread broadly and are olive to dark olive gray. The reverse is uncolored. Perithecia are abundantly produced, ostiolate, superficial, dark olive to dark olive gray and subspherical to ovate, $200\sim350\times180\sim330\ \mu$. Terminal hairs form a numerous and densely interwoven mass and olive brown, sinuous to loosely coiled, septate, minutely roughened, $2.5\sim3.5\ \mu$ wide at the base. Lateral hairs are olive brown, straight or somewhat sinuous and finally intermingled with the terminal hairs, septate, minutely roughened and $2.5\sim3.5\ \mu$ wide at the base. Asci are clavate, 8-spored. Ascospores are brown to olive brown, lemonshaped, $8.5\sim11.0\times7.0\sim8.5\ \mu$ in size and subapiculate at both ends.

Strain SANK 13479 was freshly isolated from a soil sample collected in Bangkok, Thailand. From the characters described below, the fungus was identified as *Trichoderma viride*.

Trichoderma viride PERS. *ex* FRIES, Syst. Mycol. 3: 215, 1829; RIFAI, Mycol. Pap. 116: p. 56, 1969.[4]

Colonies on potato dextrose agar grow very rapidly at 26°C and spread over the entire surface. They are velvety to fluffy, at first nearly white, then becoming yellowish green to dark green; reverse is uncolored.

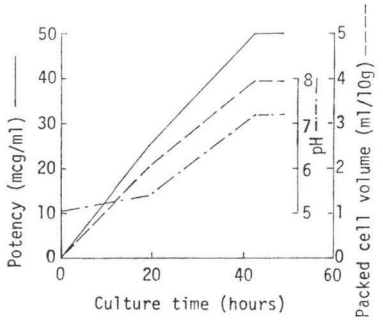
Conidiophores are erect or straggling from aerial hyphae, hyaline, septate, $3\sim4\ \mu$ in diameter and abundantly branching more or less at right angles of the main axis. Sporogenous cells are phialides, hyaline, ovate to flask-shaped, variable in size and bearing slime conidial heads at their tips. Conidia are pale green and subglobose to ovoid, $3.0\sim5.5\times2.5\sim5.3\ \mu$. Chlamydospores are produced on submerged hyphae.

At 37°C, growth-rate is somewhat the same as at 26°C.

Fermentation

One loopful growth of *Gliocladium virens* SANK 12679 on potato dextrose agar was inoculated into a 500-ml Sakaguchi flask containing 60 ml of the medium composed of glucose 2.0%, malt syrup 6.6%, peptone (Kyokuto Co., Japan) 0.1%, $NaNO_3$ 0.2% and Nissan Disfoam CB-442 (Nissan Chemical Co., Japan) 0.01%. The pH of the medium was not adjusted before sterilization. The flasks were incubated on a reciprocal shaker for 72 hours at 26°C. A 10-ml aliquot of the culture from the Sakaguchi flask was inoculated into a 2-liter Erlenmeyer flask containing 500 ml of the medium described above and incubated on a rotary shaker for 24 hours at 26°C as a seed culture. After inoculation of one liter of the seed culture into a 100-liter fermentor containing 50 liters of the same medium, fermentation was carried out for 40~50 hours with agitation (390 rev/min.) and aeration (50 liter/min.) at 26°C. Mycelial growth was expressed as the packed cell volume (ml) after centrifugation of 10 g of the culture broth at 3,000 rpm for 15 minutes. Antibiotic production during fermentation was monitored by the

Fig. 1. Fermentation of heptelidic acid in 100-liter fermentor.



disc-plate method according to the GasPak anaerobic systems (BBL division of Becton, Dickinson and Co., U.S.A.) using *Bacteroides fragilis* SANK 71176 as a test organism. An example of the time course of the fermentation in a 100-liter fermentor is shown in Fig. 1. The maximal potency of heptelidic acid, approximately 50 μg/ml, was obtained after 40~50 hours of fermentation.

Isolation

Fifty liters of the culture broth thus obtained was filtered with an aid of infusorial earth (Celite 545, Johns-Manville Products Corp., U.S.A.). The filtrate (40 liters) was adjusted to pH 3.0 with diluted hydrochloric acid and extracted once with 40 liters of ethyl acetate. The extract was concentrated to 8 liters under reduced pressure. After the antibiotic in the concentrate was transferred into 10 liters of 2% $NaHCO_3$ solution, the pH of which was adjusted to 3.0 with hydrochloric acid and the antibiotic was re-extracted twice with 5 liters of ethyl acetate each time. The extract was concentrated under reduced pressure to dryness, dissolved in 100 ml of chloroform and applied onto a silica gel column (Wakogel C-200, 25 g, Wako Pure Chem. Ind. Ltd., Japan). The column was developed with chloroform and the eluate was collected, 18 ml in each fraction. Fractions No. 5 through No. 30 were pooled and concentrated to yield 10 g of oily substance. The oily substance (8 g) was dissolved in 20 ml of the solvent mixture consisting of chloroform - ethyl acetate (1 : 1 in volume ratio) and applied on the top of Sephadex LH-20 column (700 ml) equilibrated and developed with the same solvent system as above. The eluate was collected in each 15 ml fraction and fractions No. 28 through No. 80 were pooled and removed the solvent to yield 4.8 g of oil. The oily preparation (150 mg) thus obtained was purified further by preparative TLC on silica gel (No. 5744 Merck Co. Ltd., F_{254} 0.5 mm thick) using the solvent system, *n*-hexane - benzene - acetic acid (1 : 8 : 1 in volume ratio), to yield 40 mg of colorless amorphous powder of heptelidic acid.

Physico-chemical Properties

Heptelidic acid was obtained as an amorphous powder, soluble in alkaline water, methanol, acetone and chloroform, but insoluble in acidic water. The antibiotic reacted positively to sulfuric acid, iodine and potassium permanganate-sulfuric acid. It behaved as an acidic substance on high voltage paper electrophoresis (60 volts/cm, 3 mA/cm) in 0.1 M Tris-HCl buffer at pH 7.5 for 30 minutes. The relative mobility was 0.83 when the mobility of bromophenol blue was defined as 1.0. The molecular weight and molecular formula were derived from the elementary analysis and high resolution mass spectrometry of its methyl ester derivative. The IR, PMR and CMR spectra of the antibiotic or its methyl ester derivative are shown in Figs. 2, 3 and 4, respectively. The IR spectrum indicated the existence of carboxylic acid and lactone and the PMR spectrum suggested the presence of epoxide group due to the signals of AB type protons at δ 2.59 and 3.81 ppm with coupling constant $J=5.0$ Hz. The CMR spectrum of heptelidic acid methyl ester also revealed the presence of 15 carbons in the molecule of the original antibiotic. These results as well as other physical and chemical properties are summarized in Table 1. These data coupled with its biological properties described below suggested that heptelidic acid is a new antibiotic.

From physico-chemical properties and some chemical reactions, the structure of heptelidic acid was determined as shown in Chart 1. Details of structural elucidation, however, will be reported in the subsequent paper.

Fig. 2. Infrared absorption spectrum of heptelidic acid in $CHCl_3$.

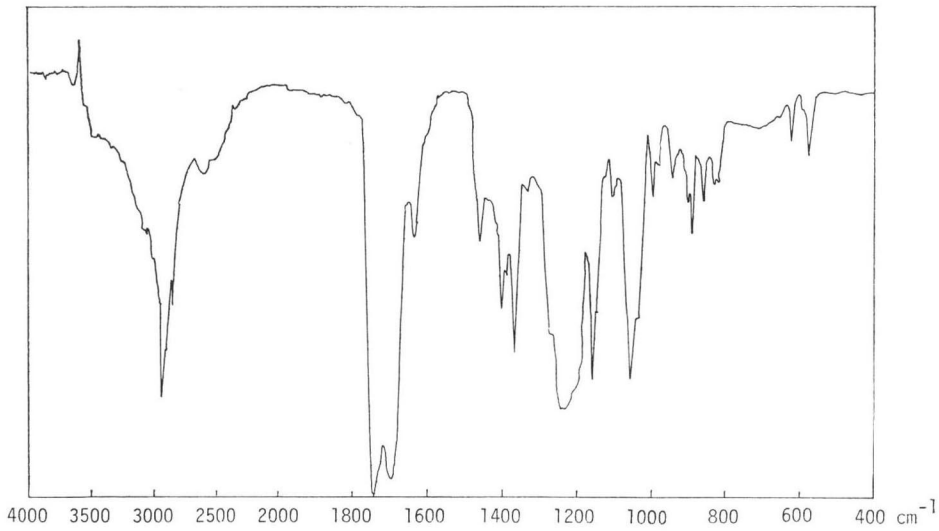


Fig. 3. PMR spectrum of heptelidic acid in $CDCl_3$. (60 MHz)

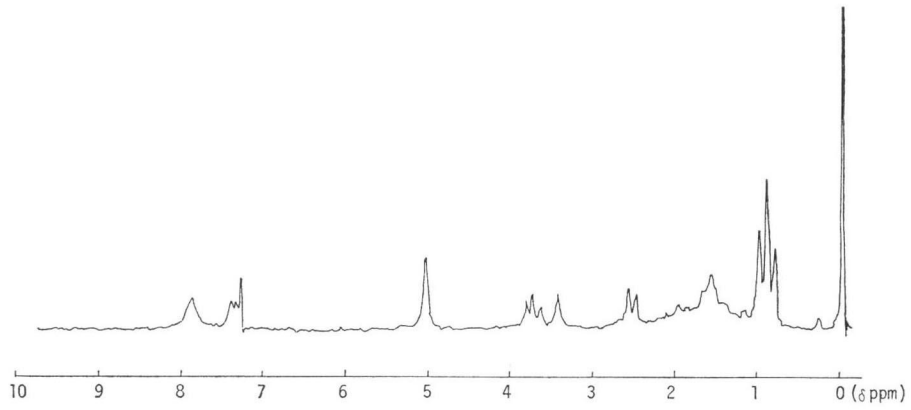
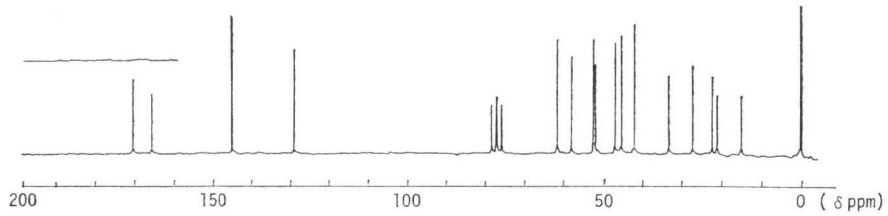


Fig. 4. CMR spectrum of heptelidic acid methyl ester in $CDCl_3$.



Biological Activity

The minimal inhibitory concentrations (MIC) of heptelidic acid against bacteria, yeast and fungi were determined by a serial two-fold agar dilution method. The results are shown in Table 2. The

media used for bacteria were heart infusion agar and GAM agar, that for yeast and some fungi, such as *Candida*, *Trichophyton*, and *Penicillium* was SABOURAUD-dextrose agar, and that for the other fungi was potato dextrose agar. The MIC were determined after incubation for 24 or 48 hours at 37°C for bacteria and for 2 or 14 days at 28°C for yeast and fungi. For the cultivation of anaerobic bacteria, the GasPak anaerobic systems were used. Heptelidic acid is active against anaerobic bacteria, especially against *Bacteroides fragilis* including several clinical isolates with MIC value of 0.4 μg/ml and also against *Propionibacterium acnes* with that of 3.13 μg/ml.

In vitro cytostatic effect of heptelidic acid on L1210 cells was determined by the method of ISHIWATA.[5] Viable cells were counted after incubation of the cells in the presence of 0.025, 0.25 and 2.5 μg/ml of heptelidic acid for 24 hours at 37°C. Mortalities of the cells treated with the antibiotic at doses of 0.025, 0.25 and 2.5 μg/

Table 1. Physico-chemical properties of heptelidic acid.

Nature	Acidic, amorphous powder
m.p.	95°C
$[\alpha]_D^{20}$	+7.7 (*c* 1.0, $CHCl_3$)
Elementary analysis (%)	Found C 63.56, H 7.28 Calcd. C 64.27, H 7.19
Molecular formula	$C_{15}H_{20}O_5$
UV λ_{max}^{MeOH}	End absorption
IR ν cm^{-1}($CHCl_3$)	3000~2500, 1740, 1700, 1640
PMR δ ppm($CDCl_3$)	0.90 3H d J=7.0 Hz 0.99 3H d J=7.0 Hz 1.3~2.0 4H m 1.55 1H m 2.15 1H m 2.60 1H m 3.55 1H d J=12.0 Hz 2.59, 3.81 2H d J=5.0 Hz 5.05, 5.08 2H d J=14.5 Hz 7.24 1H dd J=4.5, 1.5 Hz 7.90 1H s
Rf	0.41 Kieselgel 60 F_{254} 0.25 mm MERCK ($CHCl_3$ - MeOH, 9: 1)
Color reactions	Positive for H_2SO_4, I_2 and potassium permanganate

Table 2. Antimicrobial spectrum of heptelidic acid.

Test organism	Medium*	MIC (μg/ml)
Staphylococcus aureus FDA 209P JC-1	1	>100
Bacillus subtilis PCI 219	1	>100
Streptococcus faecalis S-299	1	100
S. faecalis S-299	2	25
Clostridium tetani	2	>100
Escherichia coli NIHJ JC-2	1	>100
Proteus vulgaris OX19	1	>100
Pseudomonas aeruginosa SANK 73860	1	>100
Bacteroides fragilis SANK 71176	2	0.4
B. fragilis SANK 71276	2	0.4
B. fragilis SANK 70478	2	0.4
B. fragilis SANK 70578	2	0.4
B. amylophilus 70	2	>100
B. ruminicola GA-33	2	>100
Propionibacterium acnes SANK 71976	2	3.13
Fusobacterium necrophorum 84	2	50
Candida albicans YU 1200	3	>100
Penicillium chrysogenum Q176	3	>100
Trichophyton mentagrophytes SANK 11868	3	>100
Pellicularia filamentosa SANK 22272	4	>100
Pyricularia oryzae SANK 16975	4	>100

*1: Heart infusion agar
2: GAM agar
3: SABOURAUD-dextrose agar
4: Potato-dextrose agar

ml were 0%, 20.8% and 69.6%, respectively.

The acute toxicity (LD_{50}, i.p.) of heptelidic acid in mice was 31.5 mg/kg.

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