TERRECYCLIC ACID A, A NEW ANTIBIOTIC FROM *ASPERGILLUS TERREUS*

I. TAXONOMY, PRODUCTION, AND CHEMICAL AND BIOLOGICAL PROPERTIES

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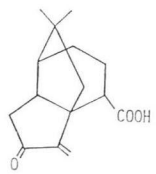
A new antibiotic, terrecyclic acid A was isolated from the culture filtrate of a new isolate of fungus, identified as *Aspergillus terreus*. The fermentation yields reached about one gram per liter of the broth. Chemical and biological characterization of the antibiotic revealed that it was a new sesquiterpene having a wide antimicrobial spectrum and antitumor activity.

A culture of *Aspergillus* strain No. 14 isolated from a soil sample picked up in the farmyard of University of Osaka Prefecture was found to produce antibiotics of broad antimicrobial spectrum. The chemical investigations described in the following paper[1] revealed that the main fraction of the antibiotics was a new antibiotic, the structure of which was a tricyclic sesquiterpene as shown in Fig. 1, and the name terrecyclic acid A was given on the basis of its producing organism and chemical structure. The present paper deals with the taxonomy of the producing organism and the fermentation, isolation, and chemical and biological characteristics of terrecyclic acid A (**I**).

Fig. 1. Structure of terrecyclic acid A(**I**).



Materials and Methods

Taxonomy of the Strain

Morphological and cultural studies were carried out using the following media. CZAPEK's agar: $NaNO_3$ 3 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, sucrose 30 g, agar 15 g, distilled water 1,000 ml. Malt extract agar: malt extract (Difco) 20 g, Polypeptone 1 g, glucose 20 g, agar 20 g, distilled water 1,000 ml. MY20 agar: Polypeptone 5 g, yeast extract 3 g, malt extract 3 g, glucose 200 g, agar 20 g, water 1,000 ml.

Fermentation Procedure

The stock culture of strain No. 14 was maintained on a CZAPEK's agar at 4°C. A loopful of spores from the stock culture was inoculated in 100 ml of the medium in a 500-ml flask. The medium composition was glucose 30 g, soybean meal 2.5 g, yeast extract 0.5 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, NaCl 0.5 g, $CaCl_2 \cdot 2H_2O$ 0.5 g, $FeCl_3 \cdot 2H_2O$ 2.0 mg, $ZnSO_4 \cdot 7H_2O$ 2.0 mg in 1,000 ml tap water and it was adjusted to pH 5.5 before sterilization. Fermentation was carried out at 30°C for 4~5 days on a

shaking machine. Antimicrobial activity was assayed by the paper disk agar diffusion method using *Staphylococcus aureus* 209P as a test organism and purified terrecyclic acid A as standard sample.

Physico-chemical Studies

The melting point was determined on a microscope hot plate and is uncorrected. The optical rotation was recorded on a JASCO DIP-SL polarimeter. The IR spectrum was obtained with a JASCO IRA-2. The UV spectrum was measured on a Shimadzu double beam spectrometer UV 180 in ethanol. The mass spectrum and the high resolution mass spectrum were obtained on a Hitachi RMU-6M mass spectrometer and a JEOL JMS D-300 mass spectrometer, respectively. The ^{1}H NMR spectrum was measured on a JEOL JMN-MH-100 spectrometer.

Antimicrobial Assay

The minimal inhibitory concentration of I was determined by the agar dilution method using the bouillon agar for bacteria and CZAPEK's agar for fungi and yeasts. Observation was made after 18 hours for bacteria and 36 hours for yeasts and fungi at 30°C following inoculation of test organisms.

Results

Taxonomy of No. 14 Strain

From the facts that No. 14 strain had a characteristic morphological feature and did not exhibit any sexual reproductive organ, it was decided that it belongs to the Genus *Aspergillus*.

1) Morphological Studies

Numerous phialide type conidia were found. Conidiophores were straight and not branched. The top of the conidiophores bulged to become vesicles with metulae and phialide.

Conidial head, cylindrical, $30\sim60\times80\sim220$ μm in length, uniform in diameter. Tan to brown, white at base.

Conidiophore, $4\sim7\times50\sim270$ μm ($100\sim200$ μm mainly). Smooth, colorless, somewhat bended.

Vesicles, $10\sim15$ μm in diameter, semispherical with metulae on upper part.

Metulae, $4\sim7\times1.5\sim2.5$ μm, cylindrical, colorless, with one or two phialide, $6\sim8\times1\sim2$ μm.

Conidia, globose or subspherical, $2\sim3$ μm in diameter, smooth, light colored.

Sclerotium was not observed. On malt extract agar or MY20 agar, globose cells, $5\sim8$ μm in diameter were formed on vegetative mycelium.

2) Cultural Characteristics

On CZAPEK's agar, growth was rapid (4.0 cm in diameter after 10 days at 25°C). The colony surface was floccose with shallow furrows white, to become yellowish and finally lemon yellow at the center. On the reverse, a dark yellowish orange pigment was formed.

On malt extract agar, growth occurred more slowly than on CZAPEK's agar (3.0 cm in diameter at the same condition). The colony surface was flat and powdery to felt-like, light tan; the formation of conidia was very good. The reverse was reddish yellow to yellowish brown; no soluble pigment was formed.

On MY20 agar, growth occurred most rapidly (7.5 cm in diameter at the same condition). The colony surface was felt-like, light tan identical to that on malt extract agar.

3) Growth Conditions

Growth range temperature, $14\sim45$°C. Optimum growth temperature, $35\sim39$°C. Growth range, pH $2\sim9$. Optimum growth, pH 3.

The above-mentioned morphological and cultural characteristics of No. 14 strain indicated that

it belonged to *Aspergillus terreus* Thom referring to the description in the textbooks of RAPER and FENNELL[2] and UDAGAWA *et al.*[3]

Production of Terrecyclic Acid A

Fermentation was performed as described in Materials and Methods. The potency of the antibiotic production was estimated at about 1,000 μg/ml at the end of the fermentation. As most of the antibiotic activity was found in the broth filtrate, the filtrate was adjusted to pH 3.0 with HCl. The active principle was extracted with ethyl acetate. The extract was concentrated *in vacuo* at 30°C and applied to a column prepared with silica gel. Active substance could be eluted with a mixture of benzene and ethyl acetate (93: 7). After recrystallization from hexane - ethyl ether, the pure antibiotic could be obtained as a white crystalline powder. Yield was about 500~600 mg from 1 liter of broth.

Chemical Properties of Terrecyclic Acid A

The crystalline form of terrecyclic acid A was obtained by recrystallization from hexane and ethyl ether, mp 122°C. The molecular formula was $C_{15}H_{20}O_3$ from the mass spectrum (Fig. 2) and elementary analysis: this was further confirmed by the high resolution mass spectrum (M^+ m/z 248.1370). The UV spectrum of **I** showed absorption maximum at 236 nm (ε 6,325). The IR spectrum is shown in Fig. 3, 3100, 2930, 1738, 1710, 1630, 1450, 1410, 1322, 1180, 1165, 940 cm^{-1}. The ^1H NMR spectrum is shown in Fig. 4. It has optical activity, $[\alpha]_D^{20}+29.1°$ (c 4, EtOH). It is easily soluble in metha-

Fig. 2. Mass spectrum of **I** (above m/z 100).

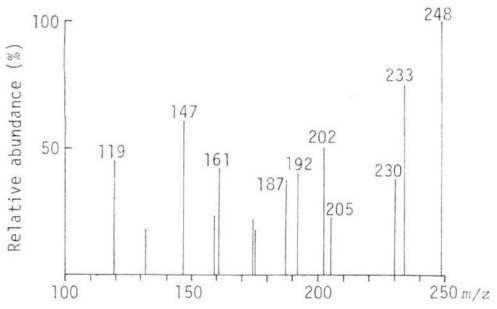


Fig. 3. IR spectrum of **I** (KBr).

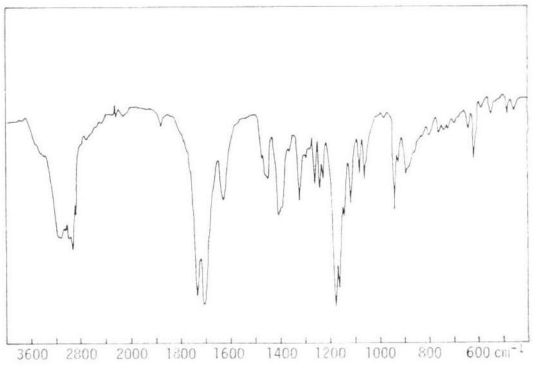
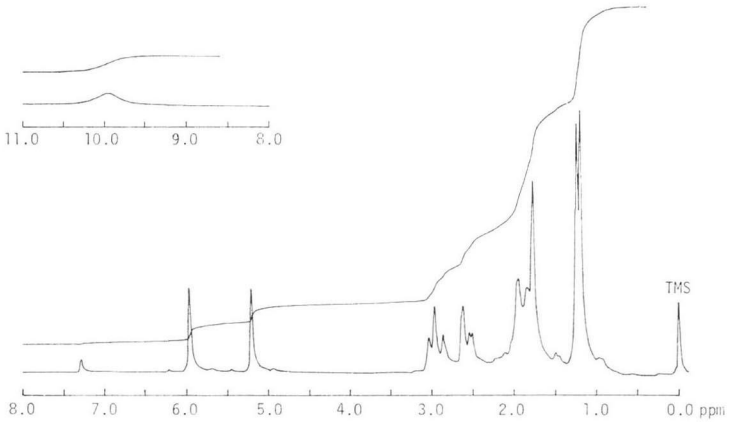


Fig. 4. ^1H NMR spectrum of **I** (100 MHz, $CDCl_3$).



nol, acetone, ethyl acetate, ethyl ether, chloroform and benzene, and hardly soluble in water and *n*-hexane. It gave a positive reaction to 2,4-dinitrophenylhydrazine and phosphotungsten reagent and decolorized aqueous potassium permanganate. The reactions of Dragendorff, ferric chloride, ninhydrin, anthrone and diphenylamine aniline reagents were negative. The Rf values on TLC using some developing solvents are shown in Table 1. Further studies on the chemical structure are described in the succeeding paper[1].

Table 1. Thin-layer chromatographic behavior of terrecyclic acid A.

Solvent system	Rf value
Benzene - methanol (80: 20)	0.32
Ethyl ether - ethyl acetate - methanol (45: 45: 10)	0.62
Ethyl acetate - methanol (93: 7)	0.66

Thin-layer chromatography; Merck silica gel plates 60 GF_{254} (0.25 mm) were used and the spots were detected under UV lamp or by spraying 2,4-dinitrophenylhydrazine solution.

Biological Characteristics

Antimicrobial Activity

Results of the examination of the antimicrobial activity of **I** are shown in Table 2. It has a wide antimicrobial spectrum of rather weak activities against Gram-positive bacteria, yeast and fungi.

When a paper disk assay was carried out using Gram-positive bacteria, the enlargement of inhibition zone was observed with the continuation of incubation time.

Acute Toxicity and Antitumor Activity

The acute toxicity (LD_{50}) of **I** by the intraperitoneal route in mice was between 125 and 63 mg/kg. As shown in Table 3, it exhibited antitumor activity against lymphocytic leukemia P388 in BDF_1 mice.

Table 2. Antimicrobial spectrum of terrecyclic acid A.

Organism	MIC (μg/ml)
Staphylococcus aureus IFO 3060	25
Bacillus subtilis IFO 12210	50
Micrococcus roseus IFO 3764	25
Escherichia coli K-12 IFO 3301	>200
Pseudomonas aeruginosa IFO 3923	200
Serratia marcescens IFO 12648	>200
Aspergillus niger IFO 4416	>200
Penicillium chrysogenum IFO 4897	200
Fusarium oxysporum IFO 5880	200
Saccharomyces cerevisiae	100
Candida albicans	150

Table 3. Effect of terrecyclic acid A on P388 leukemia.

Sample		Number of tumor cells in ascitic fluid/mouse
Control		4.5×10^7
Terrecyclic acid A	10 mg/ml	1.2×10^5
Terrecyclic acid A	1 mg/ml	1.4×10^7
Terrecyclic acid A	0.1 mg/ml	2.1×10^7
Adriamycin	0.01 mg/ml	5.4×10^5

Inoculum size of P388 was 1×10^6 cells/mouse (ip). At 1st, 2nd and 3rd day after the implantation of tumor cells, test samples were administrated to mice intraperitoneally in an injection volume of 0.2 ml per mouse. At the 4th day, the animals were sacrificed and the number of tumor cells were calculated.

Discussion

Terrecyclic acid A (**I**) had a wide antimicrobial spectrum of rather weak activities against Gram-positive bacteria, yeasts and fungi, and showed antitumor activity against lymphocytic leukemia P388. As described in the subsequent paper[1], **I** is a novel tricyclic sesquiterpene which has a close relationship to quadrone[4,5], a sesquiterpene antitumor substance produced by the same species, *Aspergillus terreus*. Accordingly, although the detailed examinations of the antitumor activity of **I** are not com-

pleted yet, the high yields of the fermentation production of the antibiotic would be promising for further studies of chemical modification.

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