ARANOROSIN, A NOVEL ANTIBIOTIC FROM *PSEUDOARACHNIOTUS ROSEUS*

I. TAXONOMY, FERMENTATION, ISOLATION, CHEMICAL AND BIOLOGICAL PROPERTIES

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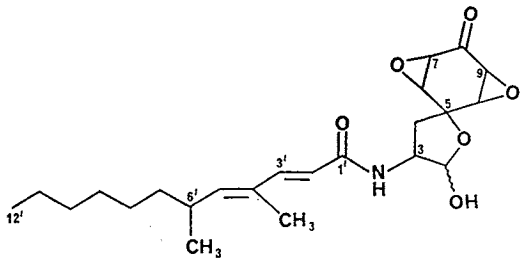
A novel antibiotic, aranorosin, was isolated from the fermentation broth of a new fungal isolate identified as *Pseudoarachniotus roseus*. The antibiotic showed a wide spectrum of activity.

During the course of our screening for novel antibiotics from fungi, a fungal strain No. Y-30,499 was isolated from a soil sample collected near Wai, Maharashtra, India and identified as *Pseudoarachniotus roseus* Kuehn[1]. We report here the isolation of a novel antibiotic, aranorosin (Fig. 1), from this strain (deposited with the German Collection of Microorganisms, Göttingen, Federal Republic of Germany; DSM No. 4151). The present paper deals with the taxonomy and fermentation of the producing strain of the fungus and the isolation, physico-chemical and biological properties of aranorosin.

Fig. 1. Structure of aranorosin.



Materials and Methods

Taxonomy of the Producing Organism

Morphological and cultural characteristics were studied on four different media at 26°C (±1°C). SABOURAUD's glucose agar: Glucose 40 g, peptone 10 g, agar 15 g, distilled water 1,000 ml. Potato glucose agar: Peeled, diced potatoes, infusion from 200 g, glucose 20 g, agar 15 g, distilled water 1,000 ml. YpSs agar[2]: Soluble starch 15 g, yeast extract 4 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, agar 15 g, distilled water 1,000 ml. CZAPEK's agar: Sucrose 30 g, $NaNO_3$ 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, agar 15 g, distilled water 1,000 ml. The pH for all these media was adjusted to 6.5 prior to autoclaving.

Fermentation

Strain Y-30,499 was maintained on SABOURAUD's glucose agar and YpSs agar. A few loopfuls of a well sporulated slant culture were transferred to 500-ml wide mouth Erlenmeyer flasks containing 100 ml of seed medium. The medium contained soluble strach 15 g, soybean meal 15 g, glucose 5 g, $CaCO_3$ 2 g, NaCl 5 g, yeast extract 2 g, corn steep liquor 1 g in 1,000 ml distilled water, and the pH was adjusted to 6.5 before sterilization. The flasks were incubated on a rotary shaker at 240 rpm for 60 hours at 26°C (±1°C) when a good growth was observed and was used as a seed culture.

The production medium contained soybean meal 20 g, glucose 30 g, $CaCO_3$ 6 g, NaCl 3 g, NH_4Cl

2.5 g, KH_2PO_4 2 g, $ZnSO_4 \cdot 7H_2O$ 0.22 mg, $CaCl_2$ 0.55 mg, $MnCl_2 \cdot 4H_2O$ 0.5 mg, $FeSO_4 \cdot 7H_2O$ 0.5 mg, $CuSO_4 \cdot 5H_2O$ 0.16 mg, $CoCl_2 \cdot 6H_2O$ 0.16 mg in 1,000 ml of distilled water. The pH of the medium was adjusted to 6.5 prior to sterilization. The medium was distributed in 200 ml amounts in 1,000-ml Erlenmeyer flasks and inoculated with 1% of the seed culture. The fermentation was carried out on a rotary shaker at 26°C (±1°C) for 72 hours.

Fermentation was also carried out in stainless steel fermentors of 150-liter capacity. The production medium (100 liters) in a 150-liter fermentor was sterilized *in situ* at 121~122°C for 32 minutes at 1.1~1.2 kg/cm² steam pressure, cooled and seeded with 1% seed. Desmophen (Bayer AG), 0.03% was used as an antifoam agent. The batch was run at 26~27°C, 100 rpm agitation, 60~80 liters/minute aeration and harvested between 66~90 hours depending upon the activity.

The activity of the fermented broth was determined by the agar diffusion assay using *Bacillus subtilis* and *Aspergillus niger* as test strains.

Isolation of Aranorosin

Aranorosin was present in both the mycelium and the culture filtrate. The filtrate (143 liters) was extracted with EtOAc (2×50 liters) and concentrated under reduced pressure at 35°C. The mycelial cake (11.3 kg) was extracted with acetone (2×30 liters). The acetone extract was concentrated under reduced pressure and the residual aqueous phase was extracted with EtOAc (2×3 liters). The EtOAc extract was concentrated as above and combined with the concentrate from the culture filtrate to give a reddish-brown crude antibiotic (196 g). This was chromatographed on silica gel (2 kg, 100~200 mesh) and eluted with $CHCl_3$ - MeOH (9.6 : 0.4) to give a semipure material (68 g). This material was chromatographed on silica gel (1.3 kg, 200~300 mesh) and eluted as above to give a further purified material (13 g). Rechromatography on silica gel (650 g, 200~300 mesh) using benzene - acetonitrile (7 : 3) for elution gave pure aranorosin (4.1 g).

Results

Morphological Characteristics

Discrete gymnothecia present but at places confluent, almost spherical, yellowish-orange to reddish-orange, 40~350 μm in diameter. Peridial hyphae hyaline, thin-walled, similar to the vegetative hyphae, 0.5~3.0 μm. Asci hyaline, ovoid to obovate, stalked or sessile, wall-evanescent, 8-spored, 7.5~11.0×9.0~12.5 μm. Ascospores are pale-yellow singly and reddish-orange in mass, conglomerate, ovoid, lenticular or elliptical in side view, spherical in face view with an outer rim, 3.5~4.5×6.0~7.5 μm, thick-walled, smooth, occasionally rough. Racquet hyphae present, 2.5~4.5 μm in diameter at the inflated areas. No asexual spores could be seen.

Cultural Characteristics

Growth on SABOURAUD's glucose agar was rapid (4.8 cm in diameter after 10 days). The colony is thin, white and cottony with extreme margin low growing. Slight furrows and ridges are seen. Reverse is white.

On potato glucose agar, the growth is slower (3.3 cm in diameter after 10 days). Colony is woolly and raised. Reverse is white and scantily furrowed.

On YpSs agar, the growth is still slower (3.0 cm in diameter after 10 days). Fruiting occurs in major growth areas in shades of orange, light orange and light yellow colors representing different stages of fruiting. Plenty of furrows and ridges are seen. Reverse is furrowed and pale-orange.

Colony on CZAPEK's agar is very slow growing (2.5 cm in diameter after 10 days). No aerial mycelium seen, low growing with lots of young fruiting seen on the surface as small light-yellow granules. No observable change on the reverse side of the plate.

The fungal strain Y-30,499 has been identified as *P. roseus* Kuehn on the basis of the above mentioned morphology and colony characteristics. This very well compares with the description of the type strain[1,3].

Physico-chemical Properties

Aranorosin is a colorless crystalline solid, mp 150°C (dec), soluble in chloroform, ethyl acetate,

Fig. 2. IR spectrum (in KBr) of aranorosin.

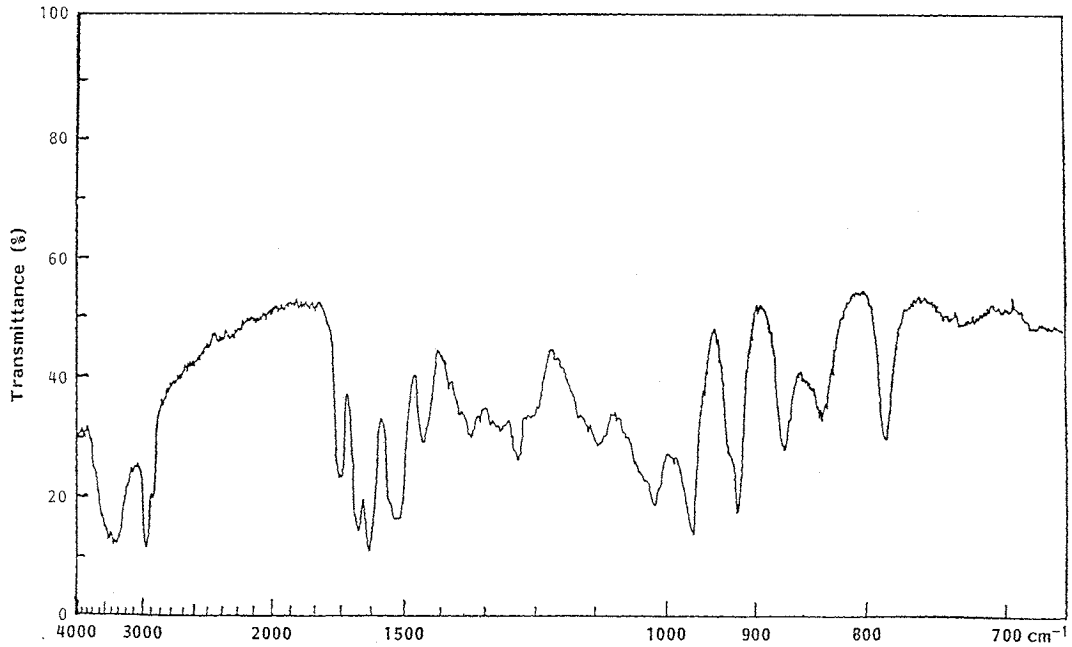


Fig. 3. 270 MHz ^1H NMR spectrum (in $CDCl_3$) of aranorosin (*H_2O peak in $CDCl_3$).

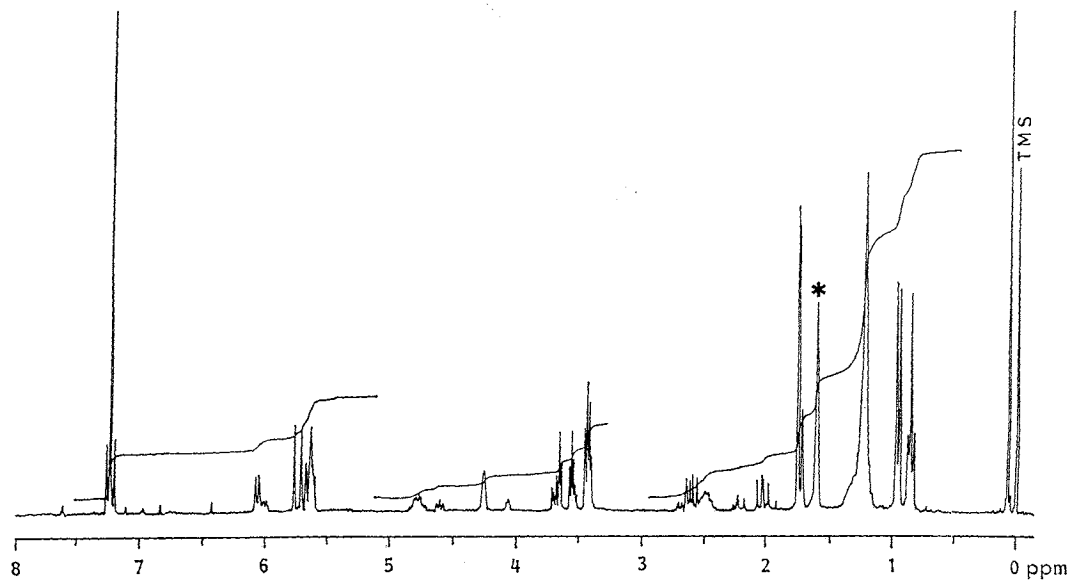


Table 1. TLC behavior of aranorosin.

Solvent system	Rf values[a]
$CHCl_3$ - MeOH (85 : 15)	0.42
Benzene - acetonitrile (6 : 4)	0.39
EtOAc	0.49

[a] Merck silica gel plates $60F_{254}$ (0.2 mm) were used and the spots were detected under a UV lamp at 254 nm.

Table 2. Antimicrobial activity of aranorosin.

Test organism	MIC (μg/ml)
Staphylococcus aureus FDA 209P	1.5
Bacillus subtilis	1.5
Micrococcus luteus	1.5
Pseudomonas aeruginosa ATCC 9027	>100
Escherichia coli Ess. 2231	>200
Salmonella typhimurium	50
Klebsiella aerogenes 1082 E	50
K. aerogenes 1522 E	>100
Enterobacter cloacae P 99	>100
E. cloacae 1321 E	>100
Candida albicans	30
Saccharomyces cerevisiae	500
Aspergillus niger	7.5
Penicillium italicum	30
Cercospora beticola	3.0
Botrytis cinerea	30
Microsporum gypseum	3.0

acetone, acetonitrile, methanol, dimethyl sulfoxide, moderately soluble in benzene and insoluble in hexane, water. Its molecular weight is 419 corresponding to the molecular formula $C_{23}H_{33}NO_6$ as deduced from the high resolution mass spectrum (found m/z 419.228, calcd 419.231). The UV spectrum in methanol showed an absorption maximum at 264 nm, no acid or alkali shift being observed. The IR spectrum is shown in Fig. 2; ν_{max} cm^{-1} 3448, 1710, 1653, 1538, 1242, 980, 844. The ^1H NMR spectrum is shown in Fig. 3. Aranorosin is optically active $[\alpha]_D^{20}$ $-2.42°$ (c 2.58, $CHCl_3$). The Rf values on TLC using different solvent systems are shown in Table 1. Further details on the structural elucidation of aranorosin will be described in the succeeding paper[4].

Biological Properties

Aranorosin possesses both antibacterial and antifungal properties. The MIC of the antibiotic required to inhibit a variety of bacterial and fungal strains are shown in Table 2. While the bacterial strains were grown on nutrient broth, the fungal strains were grown on SABOURAUD's glucose medium with the addition of 0.1% NaCl and 0.1% Na_2HPO_4. The acute toxicity (LD_{50}, ip in mice) was found to be 5 mg/kg.

Discussion

Aranorosin is a novel antibiotic produced by a fungal strain identified as *P. roseus*. This paper is the first report of an antibiotic produced by *P. roseus* and it is also the first instance where a microbial metabolite with a cyclohexanone bisepoxide moiety is being described. The antibiotic is effective against both Gram-positive and Gram-negative bacteria and against yeast and filamentous fungi.

Acknowledgments

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