RUMBRIN, A NEW CYTOPROTECTIVE SUBSTANCE PRODUCED BY *Auxarthron umbrinum*

I. TAXONOMY, PRODUCTION, ISOLATION AND BIOLOGICAL ACTIVITIES

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Rumbrin has been isolated from a fungus, *Auxarthron umbrinum*, by a screening program designed to find microorganism-produced cytoprotective substances. It was purified by use of column chromatography on silica gel, reversed phase HPLC and then isolated as fine red needles. Rumbrin prevented cell death caused by calcium overload and exhibited a potent inhibitory activity against lipid peroxidation.

Currently, therapy for ischemic heart disease is based on drugs such as β-adrenoreceptor antagonists, calcium antagonists and nitrates, which show pronounced hemodynamic effects. However, these drugs can have adverse reactions including systemic hemodynamic effects, leading to low blood pressure and peripheral edema in some patients. Recent observations that certain types of calcium antagonists such as flunarizine prevent the calcium overload in cardiac myocytes that occurs after ischemia have led to the design of a new type of anti-ischemic drugs that are cytoprotective, but have no (or little) hemodynamic effects[1].

In the course of our screening program for cytoprotective substances from microorganisms, we found that a novel substance named rumbrin (Fig. 1) was produced by a fungus strain, n13. Rumbrin prevented cell death caused by calcium overload in cells exposed to toxic concentrations of calcium ionophore A23187. Additionally, rumbrin exhibited a potent inhibitory activity against lipid peroxidation in rat brain homogenate.

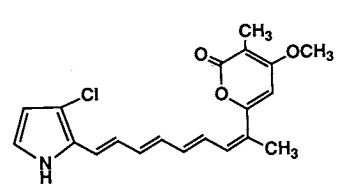
In this paper, we report the taxonomy of the producing strain, and production, isolation and biological activities of rumbrin. Physico-chemical properties and structural studies of rumbrin will be reported in the accompanying paper[2].

Materials and Methods

Chemicals

Chemicals employed were as follows: silica gel from E. Merck, Darmstadt, FRG; packed column of YMC D-ODS-7 from Yamamura Chemical Laboratories Co., Ltd., Kyoto, Japan; calcium ionophore A23187 and flunarizine from Sigma Chemical Co., St. Louis, U.S.A.; peptone from Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan; All other chemicals were of analytical grade.

Fig. 1. Structure of rumbrin.



Microorganism

Strain n13 was isolated from a soil sample collected in Australia and has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba-shi, Japan, under the accession No. FERM BP-3378.

Taxonomic Studies

For the identification of the fungus, CZAPEK's agar, potato-glucose agar, malt extract agar, and YpSs agar were used as media for plate cultures.

Fermentation

The seed medium consisted of glucose 2.5%, soy bean meal 1.5%, dry yeast 0.2% and $CaCO_3$ 0.4% (pH being adjusted to 7.2 before autoclaving), and the production medium consisted of glucose 2.0%, peptone 0.5%, KH_2PO_4 0.5%, $MgSO_4 \cdot H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.05% and agar 0.1% (pH adjusted to 6.0 before autoclaving).

Assays for Biological Activity

Cytoprotective activity against cell death caused by calcium overload was examined by a modified method of HIRAI *et al.*[3], as follows. 3T3-Swiss albino cells (mouse embryo cell line) were inoculated into each well of a 96-well collagen-coated plate containing 100 μl of DULBECCO's modified minimum essential medium supplemented with 10% fetal bovine serum (D-MEM). After overnight incubation at 37°C in an atmosphere of 5% CO_2 in air, the culture supernatant was discarded and the confluent cells were given 100 μl of D-MEM containing various concentrations of samples and 50 μl of D-MEM containing 5×10^{-5} M calcium ionophore A23187. After 90 minutes incubation at 37°C in an atmosphere of 5% CO_2 in air, cell viability was measured as the value of A_{570} by colorimetric MTT assay[4]. The percent of cell viability was calculated by the formula $[(C-B)/(A-B)] \times 100$, where A is the value of A_{570} in the system without A23187, B is that with A23187 and C is that with A23187 and a sample. The concentration range of a sample required for more than 50% of cell viability was determined.

Inhibitory activity against lipid peroxidation in rat brain homogenate was measured according to the method of KUBO *et al.*[5] in the presence of ascorbic acid (100 μM).

Results

Taxonomic Characterization of the Producing Strain

The cultural and physiological characteristics of strain n13 grown at 25°C for 14 days are summarized in Table 1. The organism grew on various media and formed orange colonies (20~40 mm in diameter). The colony surface was wooly, and the color of the reverse side of colony was reddish-orange under almost all conditions employed.

The morphological properties of strain n13, when cultured on CZAPEK's agar at 25°C for 11 days are summarized in Table 2. Under this condition, the appendages and the ascocarps were produced and the ascospores were spherical or oblate and rough as shown in Fig. 2.

From the above characteristics, we determined that strain n13 is a strain of *Auxarthron umbrinum*[6],

Table 1. Cultural characteristics of strain n13.

Medium	Growth (colonyl size)	Color of colony	The color of the reverse side of colony	Soluble pigment
Malt extract agar	Wooly (20~30 mm)	Orange	Orange	None
CZAPEK's agar	Wooly	Pale yellow	Pale yellow	None
Potato-glucose agar	Wooly (30~40 mm)	Orange	Orange	None
YpSs agar	Wooly	Orange	Yellow	None

Fig. 2. (a) Micrograph of ascocarps and appendages of strain n13 (× 100), (b) Scanning electron micrograph of ascospores of strain n13 (Bar represents: 0.5 μm).

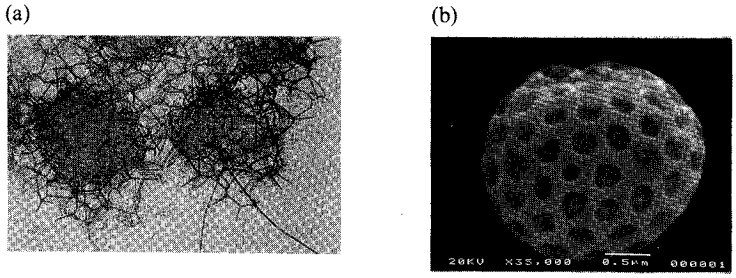


Table 2. Morphological properties of strain n13.

Conidiospore	None
Ascocarp	Yellow-brown, spherical or oblate, reticuloperidium with hyphae
Appendage	Brown, light colored tips, rare branches, smooth, straight or slightly uncinate tips
Asci	Spherical or oblate, octave spore
Ascospore	Rough, spherical or oblate

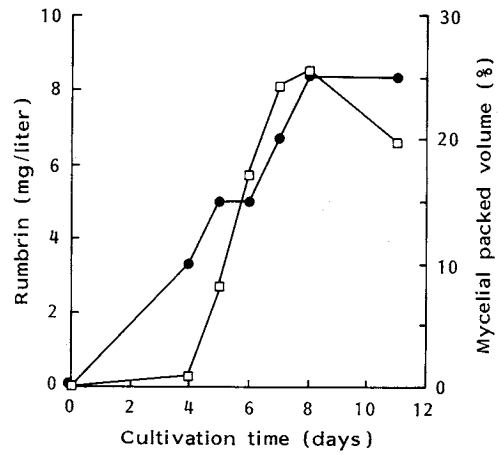
Table 3. Biological activities of rumbrin.

	Rumbrin	Flunarizine ·2HCl
Cytoprotective activity* (μg/ml)	1.3~40	1.3~80
Inhibitory activity against lipid peroxidation IC_{50}(μg/ml)	0.47	11.5

* Concentration range required for more than 50% of cell viability.

Fig. 3. Time course of rumbrin production by *Auxarthron umbrinum* n13.

□ Rumbrin, ● mycelial packed volume.



and it was designated as *Auxarthron umbrinum* n13.

Production and Isolation of Rumbrin

Strain n13 was inoculated into 100 ml of the seed medium in a 500-ml Erlenmeyer flask, and cultured at 25°C for 5 days on a rotary shaker (180 rpm) to obtain a seed culture. Three-ml portions of this seed culture was inoculated into 100 ml portions of the production medium in 500-ml Erlenmeyer flasks and cultivated on a rotary shaker (180 rpm) at 25°C. The time course of the production is shown in Fig. 3. The maximum peak of rumbrin production was obtained at 8 days cultivation and thereafter the productivity decreased.

The cultured broth (5 liters) was filtered and the mycelial cake was extracted with MeOH (2 liters). After removal of MeOH, this extract and broth filtrate were combined and extracted with EtOAc (5 liters). The organic layer was evaporated to dryness to give a residue (232 mg). This residue was chromatographed on a column of silica gel (150 ml) with $CHCl_3$. The active eluate was concentrated under reduced pressure to give crude rumbrin (100 mg), which was further purified by reversed phase HPLC, using a packed column of YMC D-ODS-7 (2 × 25 cm, flow rate 6.5 ml/minute) and 80% aqueous MeOH as the developing

solvent. After concentration of the active fraction, the resultant red powder (27 mg) was subjected to crystallization in MeOH at 4°C overnight to obtain fine red needles of rumbrin (10 mg). The purity of each preparation was confirmed by TLC and HPLC.

Biological Activities of Rumbrin

As shown in Table 3, rumbrin was as active as flunarizine in cytoprotective action. In addition, rumbrin showed much higher inhibitory activity against lipid peroxidation than flunarizine.

Rumbrin had no antimicrobial activity at 1 mg/ml against *Bacillus subtilis* and *Candida albicans*. It showed low toxicity; there was no death after ip injection in mice of 200 mg/kg.

Discussion

Flunarizine is one of the well-known brain protective agents, and it seems likely that a part of its anti-ischemic effect is the result of the prevention of membrane lipid peroxidation associated with abnormal calcium influxes into cells[5]. Our results suggest that rumbrin has a quite similar mode of action to that of flunarizine. Therefore rumbrin is expected to be useful for myocardial and cerebral ischemia[1,5], by the alleviation of tissue damage due to calcium overload and/or peroxidative disintegration of cell membranes. The *in vivo* activity of rumbrin is now under study.

References

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