RHIZOPODIN, A NEW COMPOUND FROM *Myxococcus stipitatus* (MYXOBACTERIA) CAUSES FORMATION OF RHIZOPODIA-LIKE STRUCTURES IN ANIMAL CELL CULTURES

PRODUCTION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES†

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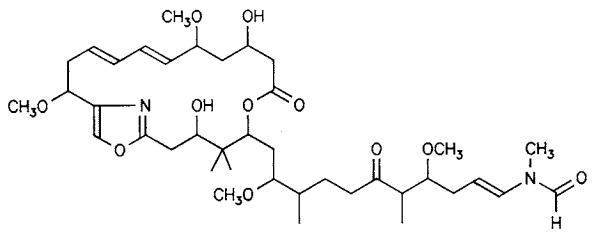
A new cytostatic compound, rhizopodin, was isolated from the culture broth of the myxobacterium, *Myxococcus stipitatus*. The compound inhibited growth of various animal cell cultures without killing the cells. The ID_{50}, measured by an MTT assay, was 12~30 ng/ml, depending on the cell line. Especially cells growing fibroblast-like showed typical morphological changes. They became larger and within hours formed long branching and reticular runners. These morphological changes were irreversible. Rhizopodin suppresses bleb formation in K-562 cells, and therefore could act by interacting with protein phosporylation.

Myxobacteria are a rich source of antibiotics[1]. Most of these compounds were found in screening programs with bacteria and fungi as indicator organisms. We want to report herein on a new substance that was found by screening with mammalian cell cultures. The compound, which we named rhizopodin, inhibited growth of animal cells and caused striking morphological alterations of fibroblast cultures. The structural elucidation showed that rhizopodin is a macrocyclic lactone with an integrated oxazole ring and a C-11 side chain ending with an *N*-formyl group (Fig. 1). Rhizopodin is chemically related to the scytophycins[2] from the cyanobacterium *Scytonema pseudohofmanni*, to sphinxolide[3] from a pacific nudibranch, and also to a group of compounds, called ulapualides[4], kabiramides[5] and halichondramides[6], found in nudibranch egg masses and in the sponge *Halichondria* sp., respectively. While the macrolides of the second group contain three oxazole rings, the first two compounds possess no oxazole at all. All

Fig. 1. The structure of rhizopodin[7].



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these related macrolides have an identical or slightly modified side chain. In this paper we describe the production, isolation, and the physico-chemical and biological properties of rhizopodin. The structure elucidation will be published elsewhere[7].

Screening Assay

Methanolic cell extracts from myxobacterial cultures and methanolic eluates of Amberlite XAD resin, which had been added to the cultures, were serially diluted in 3-fold steps with cell culture medium in titerplates and incubated with the mouse fibroblast cell line L929 (ATCC CCL 1) or the human leukemia cell line K-562 (ATCC CCL 243). The inoculum was 6,000 cells/180 μl culture volume. The media used for these cultures were DULBECCO's modified EAGLE medium with high glucose and RPMI 1640 medium, respectively (Gibco, Life Technologies). Both media were supplemented with 10% newborn calf serum. The titerplates were incubated under 10% CO_2 at 37°C. Growth and morphological changes were observed under an inverse microscope. Growth and metabolic activity were also determined by an MTT assay[8], which measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to its insoluble formazan. Rhizopodin was discovered in the Amberlite XAD resin eluate of strain Mx f164 because of the morphogenetic effects mentioned above.

Microorganism and Culture Conditions

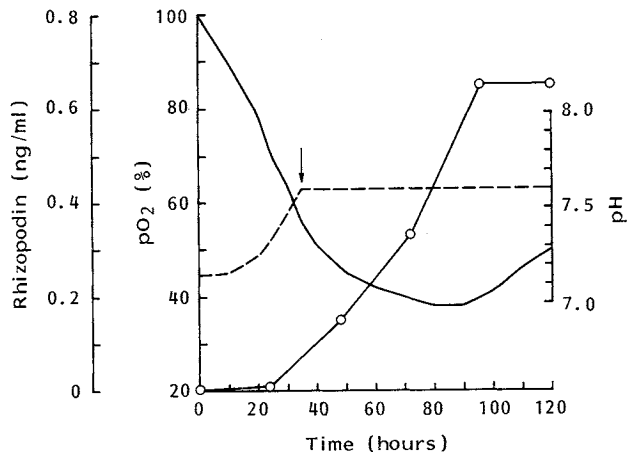
The producing organism is *Myxococcus stipitatus*, strain Mx f164, isolated in 1981 at the GBF from a soil sample collected on San Andrés Island, Columbia. The strain was deposited at the DSM (depository number DSM 6807). It was grown in standard peptone liquid medium (MD1 l.m.: peptone from casein, pancreatically digested (Marcor, Hackensack, N.J.) 0.3%, $MgSO_4 \cdot 7H_2O$ 0.2%, $CaCl_2 \cdot 2H_2O$ 0.05%, pH 7.2). Batch cultures of 100 or 500 ml in 250-ml or 1,000-ml Erlenmeyer flasks, respectively, were incubated at 30°C on a gyratory shaker at 160 rpm for 3~5 days.

Production

Rhizopodin production on a larger scale was performed in standard MD1 liquid medium. For example,

Fig. 2. Fermentation of *Myxococcus stipitatus* Mx f164 in a 365-liter draft tube reactor with a kaplan turbine stirrer (300 liters culture volume, 200 rpm, aeration rate 30 liters/minute).

○ Rhizopodin, — pO_2, --- pH.



After 35 hours the pH was regulated by adding 5% H_2SO_4 (arrow) and thus kept at 7.6.

Fig. 3. 150 MHz ^{13}C NMR spectrum of rhizopodin in CD_3OD (Bruker WM-600 spectrometer).

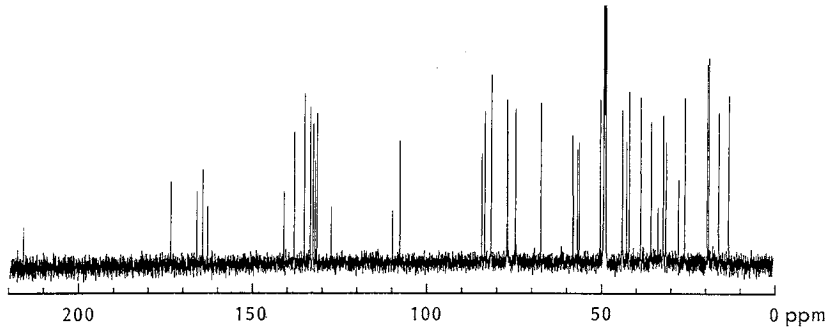
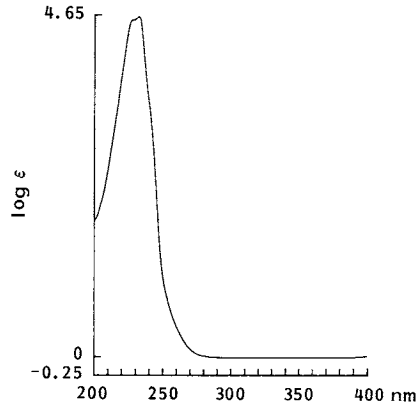


Fig. 4. UV spectrum of rhizopodin in methanol.



500 ml of culture grown for 3 days on a gyratory shaker was inoculated into 10 liters in a 15-liter fermentor with a flat blade turbine stirrer. After 3 days the content of the first fermenter was inoculated into a second one with 300 liters medium, to which 1% (v/v) adsorbent resin Amberlite XAD-16 (Rohm & Haas, Frankfurt) was added. Fig. 2 shows a fermentation in a 365-liter bioreactor (Giovanola Frères SA, Monthey, Switzerland). Both fermentors were kept at 30°C and agitated at 150 and 200 rpm, respectively. The aeration rate was 0.1 volume air per volume culture and minute. The pH increased during the fermentation and was kept at 7.6 by titration with 5% H_2SO_4. The rhizopodin production was followed by the cell assay and HPLC analysis. Harvest was after 5 days. The adsorbent resin was separated from the culture broth by passage through a process filter (210 μm pore size).

Isolation

Rhizopodin could be eluted from the resin by methanol and was further purified by column chromatography on Sephadex LH-20 (solvent: methanol) and silica gel RP-18 (solvent: methanol - water (8 : 2)). From a 300-liter fermentation 150 mg rhizopodin was obtained. Rhizopodin was quantitatively determined by HPLC (column 250 × 4 mm, HD-Sil-C18, 7 μm (Knauer, Bad Homburg); solvent: methanol - water (8 : 2); flow rate 1.0 ml/minute, Rt 6.7 minutes; detection: diode-array).

Physico-chemical Properties

Rhizopodin is soluble in methanol, acetone and ethyl acetate, sparingly in chloroform and ethyl ether, and almost insoluble in water and hexane. In TLC on silica gel 60 F_{254} with dichloromethane - methanol (9 : 1) as the solvent the Rf value was 0.5. After spraying the plates with vanillin/sulfuric acid reagent and heating to 120°C, rhizopodin gave brown spots. ^{13}C NMR (Fig. 3) and high resolution FAB (−) mass spectroscopy gave a molecular formula of $C_{39}H_{62}N_2O_{11}$ and a mass of 734 g/mol. The doubling of ^{13}C NMR signals (Fig. 3) is caused by restricted rotation of the *N*-formyl group. Detailed data will be published with the structure elucidation[7]. The optical rotation of rhizopodin was $[\alpha]_D$ −53.6° (*c* 1.0, MeOH). The

Fig. 5. IR spectrum of rhizopodin in chloroform.

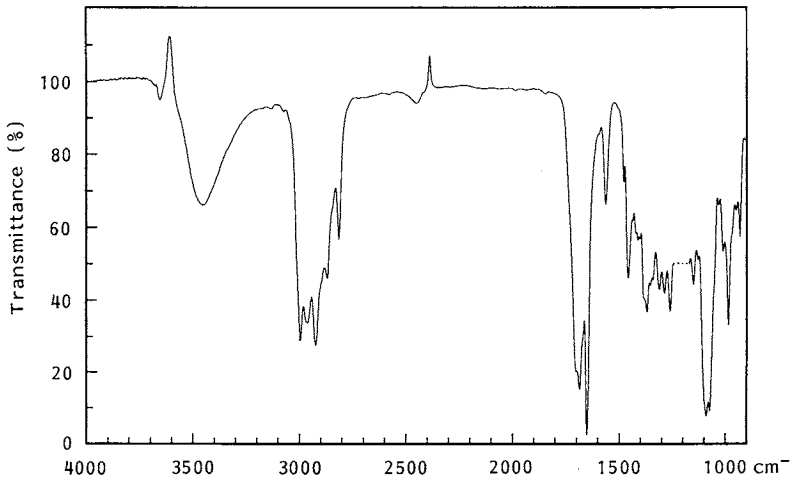


Table 1. Antimicrobial activity of rhizopodin.

	Test organism	Diameter of inhibition zone[a,b] (mm)
Gram-negative bacteria	*Escherichia coli* DSM 498[c]	0
	Pseudomonas aeruginosa DSM 1117	0
Gram-positive bacteria	*Bacillus subtilis* DSM 10	0
	Micrococcus luteus GBF[d]	0
	Nocardia flava Zü 13086[e]	0
	Staphylococcus aureus GBF	0
Yeasts	*Candida albicans* CBS 1893[f]	0
	Debaryomyces hansenii DSM 70238	0
	Hansenula anomala DSM 70263	0
	Kloeckera corticis GBF	0
	Lipomyces lipofer GBF	0
	Metschnikowia pulcherrima DSM 70321	0
	Nadsonia fulvescens CBS 2596	0
	Pichia membranaefaciens DSM 70366	0
	Saccharomyces cerevisiae BT 27C-2AYGSC[g]	11
	Schizosaccharomyces pombe Tü 501[h]	0
	Torulopsis glabrata DSM 70398	7
Filamentous fungi	*Aspergillus niger* DSM 823	0
	Athelia rolfsii DSM 63030	11
	Botrytis cinerea DSM 877	0
	Cercospora beticola DSM 62107	27
	Fusarium oxysporum DSM 2018	0
	Mucor hiemalis DSM 2655	18
	Pythium debaryanum DSM 62946	0
	Rhizopus oryzae DSM 905	11
	Trichoderma koningii DSM 63060	0
	Ustilago zeae DSM 3121	11

[a] Determined by agar diffusion test using paper discs of 6 mm diameter with 20 μg rhizopodin.
[b] The organisms were tested on standard complex media.
[c] Deutsche Sammlung von Mikroorganismen.
[d] Collection at the GBF.
[e] Collection University Zürich.
[f] Centraalbureau voor Schimmelcultures Baarn.
[g] Yeast Genetic Stock Center Berkeley.
[h] Collection University Tübingen.

UV spectrum of rhizopodin dissolved in methanol was recorded with a Hitachi U 3200 spectrophotometer (Fig. 4: λ_{max} 231 nm; ε 44,700). The IR spectrum was measured with a Nicolet 20 DXB FT-IR spectrometer (Fig. 5).

Biological Activity

As shown in Table 1, rhizopodin had no effect on bacteria and only a weak one on a few fungi, but was highly effective in animal cell cultures. It was not mutagenic in an Ames assay[9] at concentrations up to 50 μg/ml.

Concentration-dependent inhibition curves of various mammalian cell lines, measured by the MTT assay, are shown in Fig. 6. The IC_{50} was 30 ng/ml for BHK (GBF, hamster), 12 ng/ml for CHO (DSM ACC 126, hamster), and 15 ng/ml for L929 and K-562 cells. At rhizopodin concentrations that were equal

Fig. 6. Concentration dependence of growth inhibition by rhizopodin for different cell lines measured after five days.

○ L929, mouse, △ CHO, hamster, ▽ K-562, human, □ BHK, hamster.

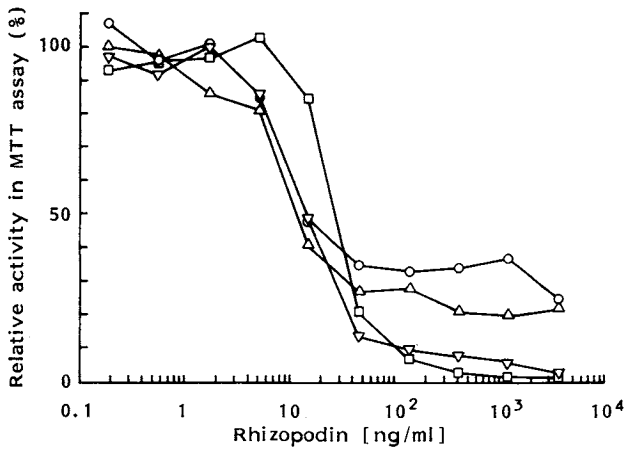
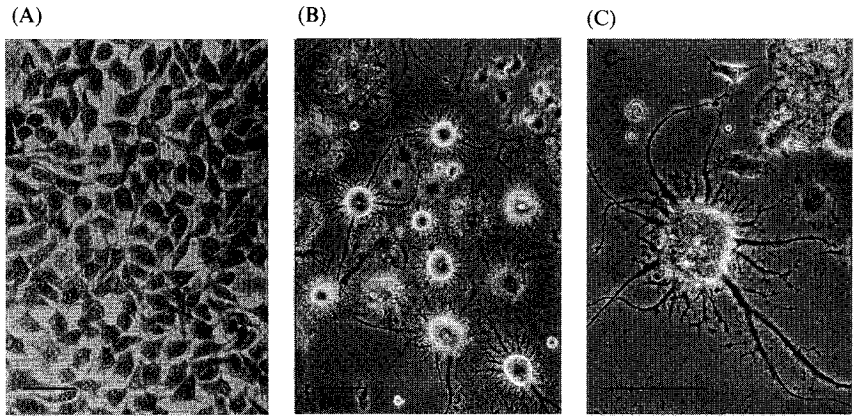


Fig. 7. Morphological differentiation of L929 mouse cells in the presence of rhizopodin.

(A) Normal fibroblast cells (control). (B, C) Cells with 50 ng/ml rhizopodin after 2 days at different magnification. Scale bars, 100 μm.



or higher than the IC_{50} values, adherently growing cells underwent typical morphological changes. Within hours they became larger and formed long, branching and reticular runners, which resembled the rhizopodia of certain protozoa. These specific changes were observed with all adherently growing cell lines tested (BHK, CHO, L929, HeLa (GBF), Vero (ATCC CCL 81)), but with BHK and HeLa cells they were weakly expressed and occured only in a small concentration range (40~140 ng/ml). The most striking alterations were observed with the L929 mouse fibroblasts (Fig. 7) and the CHO hamster ovary cells, where the effect occured in a range between 15 and 4,000 ng rhizopodin per ml. Both lines also showed a higher metabolic activity at higher rhizopodin concentrations (Fig. 6).

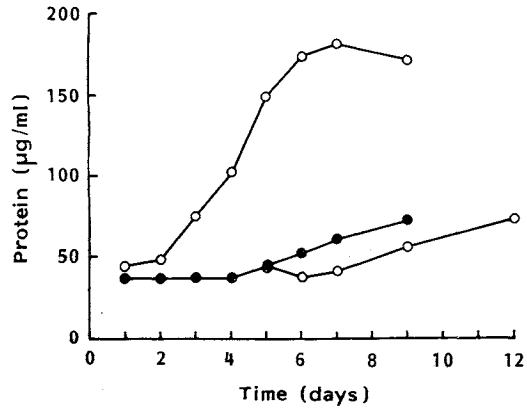
Fig. 8 shows the growth kinetics of L929 mouse fibroblasts in the presence and absence of rhizopodin. The cells were cultivated in 24-well plates with 0.75 ml/well. The inoculum was 50,000 cells/ml. As a parameter of growth, the protein amount was measured[10]. At harvest, the cells were frozen in their wells. After thawing, the cells were disintegrated by adding 0.4% sodium hydroxide (50 μl/well). After 10 minutes, protein reagent was added (2×2 ml), transferred to a cuvet, and the absorbance was measured at 595 nm.

Rhizopodin inhibited growth from the beginning, but not completely. Microscopical observations showed that there were always some cells left in the culture that did not undergo morphological alterations and apparently continued to propagate. The number of the cells that had formed rhizopodia-like structures did not further increase after 2 days.

Rhizopodia production was not reversible. If the medium was exchanged for fresh medium without rhizopodin, rhizopodia cells did not return

Fig. 8. Growth of L929 mouse cells in the presence of rhizopodin.

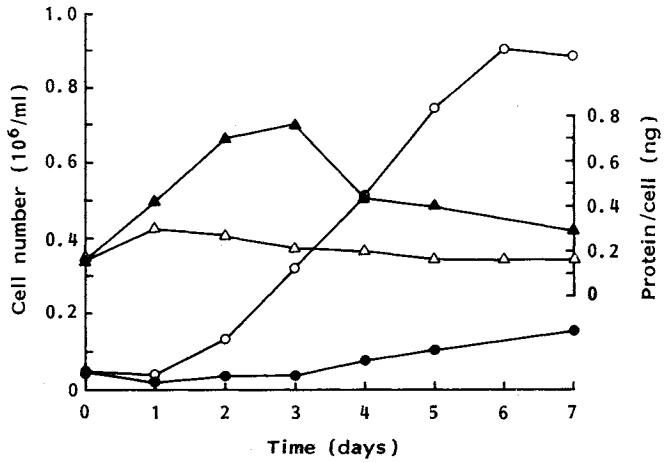
● Rhizopodin (50 ng/ml), ○ without rhizopodin.



After 5 days the medium of some cultures was exchanged for fresh medium without the inhibitor.

Fig. 9. Growth of K-562 human leukemia cells in the presence of rhizopodin.

Closed symbols: 50 ng/ml rhizopodin, open symbols: control; ●, ○ cell number, ▲, △ protein/cell.



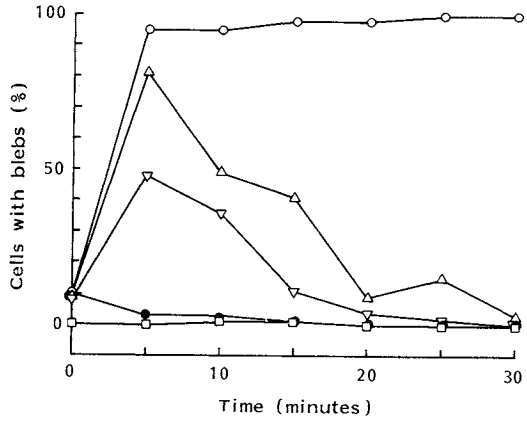
to the normal state, and no faster growth of the culture was observed. When the unaltered cells finally had formed a continuous monolayer after three weeks, the rhizopodia cells could still be detected.

The lymphoblast-like K-562 cells did not form runners in the presence of rhizopodin. They also became larger, but developed big bubbles instead of runners. As with the mouse cells, cell propagation was inhibited from the beginning, but again not completely (Fig. 9). Rhizopodin treated cells were found to have higher protein contents. After three days incubation the protein content per cell reached almost three times the value of the control.

K-562 cells can be induced by phorbol esters to form blebs[11]. The bleb formation was correlated with the activity of protein kinase C (PKC) and could be suppressed by PKC inhibitors[11]. The induction was done in 24-well plates with 100,000 cells/0.5 ml medium, to which 1 μmol/liter phorbol dibutylate was added. When rhizopodin was given to K-562 cells together with the phorbol ester, the cells formed blebs within five minutes. But microscopic observations showed that these blebs separated from the cell surface, and after half an hour almost no cells with blebs could be observed (Fig. 10). The ED_{50} for rhizopodin was 300 ng/ml. When the cells were incubated with rhizopodin for half an hour before they were induced with phorbol ester, no bleb formation could be observed. The ED_{50} was 40 ng then. The same result was found, when staurosporine, a potent inhibitor of PKC, was added to K-562 cells simultaneously with the phorbol ester. The ED_{50} was 1 ng/ml.

Fig. 10. Influence of rhizopodin (10 μg/ml) and staurosporine (0.1 μg/ml) on bleb formation by K-562 cells induced by phorbol 12,13-dibutylate (PDBu; 1 μmol/liter).

○ Control, □ staurosporine and PDBu simultaneously, △ rhizopodin and PDBu simultaneously, ▽ rhizopodin 5 minutes before PDBu, ● rhizopodin 30 minutes before PDBu.



Cells were counted under the microscope.

When staurosporine was added to L929 cells at different concentrations (up to 0.5 μg), we did not observe the morphological alterations typical for rhizopodin. The MIC for cell growth in the screening assay was 0.25 ng/ml. Neither did other compounds known to interfere with structures of the cytoskeleton, like colchicine and vinblastine, show effects resembling those of rhizopodin.

Discussion

More than half a dozen different basic structural types of antibiotics have been isolated from the genus *Myxococcus* (for a review see REICHENBACH *et al.*[1]). For *M. stipitatus* the fluorescent compound stipiamide[12] (phenalamide[13]) has been reported, which is a straight chained polyene and thus completely different from rhizopodin. But both compounds seem to be specific for *M. stipitatus*. Because of the striking effect of rhizopodin on the morphology of animal cells, we suppose that rhizopodin interferes with mechanisms that are connected to the cytoskeleton. The results with the K-562 cells seem to indicate that this could be *via* an alteration of protein phoshorylation. But the mechanism appears to be different from that of staurosporine, because this compound inhibited bleb formation of K-562 cells directly without a preincubation and did not produce the morphological changes of L929 cells typical for rhizopodin.

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