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Aspochalamins A~D and Aspochalasin Z Produced by the Endosymbiotic Fungus *Aspergillus niveus* LU 9575

I. Taxonomy, Fermentation, Isolation and Biological Activities

KLAUS GEBHARDT[a], JUDITH SCHIMANA[a], ALEXANDRA HÖLTZEL[b], KONRAD DETTNER[c], SIEGFRIED DRAEGER[d], WINFRIED BEIL[e], JOACHIM RHEINHEIMER[f] and HANS-PETER FIEDLER[a,*]

[a] Mikrobiologisches Institut, Universität Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany
[b] Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 18, D-72076 Tübingen, Germany
[c] Lehrstuhl für Tierokologie II, Universität Bayreuth, Universitätsstr. 30, D-95400 Bayreuth, Germany
[d] Institut für Mikrobiologie, Technische Universität Braunschweig, Spielmannstr. 7, D-38106 Braunschweig, Germany
[e] Institut für Pharmakologie, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany
[f] ZHP, BASF AG, D-67056 Ludwigshafen, Germany

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Aspochalamins A~D, a family of new cytochalasan antibiotics have been isolated from *Aspergillus niveus*, an endosymbiotic fungus isolated from the gut of a woodlouse belonging to the family *Trichoniscidae*. Besides aspochalamins, aspochalasin Z, a new member of the aspochalasin family, as well as the known mycotoxins aspochalasin D and citreoviridins A/C and B were isolated from the mycelium. Aspochalamins showed cytostatic effects towards various tumor cell lines and a weak antibacterial activity against Gram-positive bacteria.

During our screening program for detection of novel bioactive metabolites derived from endosymbiotic microorganisms isolated from arthropod hosts[1,2] *Aspergillus niveus* LU 9575 was investigated regarding the diversity of secondary metabolites. The fungus was isolated from the gut of a woodlouse, a member of the isopod family *Trichoniscidae*. A methanol - acetone extract of the mycelium from a 24-day stationary culture was analysed by gradient reversed-phase HPLC coupled with a diode array monitoring system (HPLC-DAD). The UV-visible spectra of the resulting peaks were compared with those of more than 750 reference compounds, mostly antibiotics, stored in our HPLC-UV-Vis-Database[3]. The comparison of the UV-visible spectra resulted in the discovery of at least five presumed novel metabolites. Four of them with retention times of 11.6, 12.2, 12.8 and 13.8 minutes possessed nearly similar UV-Vis spectra and were named aspochalamins A~D. A compound with the retention time of 7.5 minutes, a new member of the aspochalasin family, was named aspochalasin Z. Besides these novel metabolites, several already known compounds, *e.g.* aspochalasins B and D[4,5] and citreoviridins A/C and B[6], were also detected in the mycelium extract.

This paper deals with the taxonomy of the producing strain, its fermentation, and the isolation and biological activities of the metabolites isolated from *Aspergillus niveus* LU 9575. Investigations of the chemical structures are reported elsewhere[7].

* Corresponding author: hans-peter.fiedler@uni-tuebingen.de

Materials and Methods

Organisms

Aspergillus niveus LU 9575 was isolated from the intestine of a woodlouse, collected near Königsheide, Germany. Prior to dissection the isopod was decontaminated in 70% ethanol to avoid contamination of the isolation plates with microorganisms attached to the exterior surface of the animal. Then the gut was prepared under the light-microscope and the content incubated using humic acid - vitamin agar[8].

Taxonomic studies of strain LU 9575 were performed according to the method of RAPER & FENNELL[9]. The strain was kept on an agar plate at 6°C in a medium consisting of glucose 0.4%, malt extract (Oxoid) 1%, yeast extract (Oxoid) 0.4%, and agar 1.5% in tap water (pH 5.5). It is deposited in the culture collection of BASF, Ludwigshafen, Germany.

Microbial strains for testing biological activity spectra were obtained from DSMZ, ATCC, CBS and the culture collection of the Mikrobiologisches Institut, Universität Tübingen.

Fermentation

Strain LU 9575 was cultivated in nine 3-liter penicillin flasks, each filled with one liter of medium consisting of starch 1%, glucose 1%, cornsteep powder (Marcor) 0.25%, Bacto peptone 0.5%, yeast extract (Oxoid) 0.2%, NaCl 0.1% and $CaCO_3$ 0.3% in tap water. The pH was adjusted to 7.3 prior to autoclaving. Each penicillin flask was inoculated with a quarter of a well-grown agar plate (90 mm diameter) covered with strain LU 9575. The medium for the seed culture consisted of yeast extract (Oxoid) 0.4%, malt extract (Oxoid) 1%, glucose 0.5% and agar 1.5% in tap water (pH 5.5). Cultivation was carried out for 24 days at 27°C until the mycelium covered the whole penicillin flask and the surface color turned yellow.

Isolation

The mycelium was separated by filtration and extracted three times with MeOH - acetone (1 : 1). The extract was concentrated to dryness, dissolved in a small volume of CH_2Cl_2 - MeOH (3 : 1), and applied to a silica gel column (400×26 mm, silica gel 60, 40~63 μm; Merck) with CH_2Cl_2 - MeOH as eluent. The metabolites were desorbed with a linear gradient starting with CH_2Cl_2 up to CH_2Cl_2 - MeOH (85 : 15) within 4 hours at a flow rate of 4 ml/minute (medium pressure pump model 381, gradient controller model 388; Büchi). The separation resulted in three main fractions containing aspochalamins, aspochalasins and citreoviridins, respectively. Further purification was achieved by Sephadex LH-20 column chromatography (900×25 mm) in MeOH. Single metabolites were obtained after preparative reversed-phase HPLC using a stainless steel column (250×16 mm) filled with 10-μm Nucleosil-100 C-18 (Maisch) and linear gradient elution using H_2O - acetonitrile in the case of aspochalamins and aspochalasin Z, and with isocratic elution using H_2O - acetonitrile (1 : 1) in the case of citreoviridins, at a flow rate of 20 ml/minute. The preparative system consisted of two high-pressure pumps (Sepapress HPP-200/100; Kronwald), gradient unit (Sepacon GCU-311) and a Valco preparative injection valve (Mod. 6UW; VICI) with a 5-ml sample loop. The UV absorbance of the eluate was monitored simultaneously at 260 nm and 280 nm using a Gilson spectrophotometer model 116 equipped with a preparative cell. After lyophilisation aspochalamins and aspochalasins D and Z were obtained as white powders, and citreoviridins as yellow powders.

HPLC-DAD Analysis

The chromatographic system consisted of a HP 1090M liquid chromatograph equipped with a diode array detector and HP Kayak XM 600 ChemStation (HPLC software revision A.08.03; Agilent Technologies). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360, 435 and 500 nm. Spectra were measured from 200 to 600 nm with a 2-nm step and a sampling rate of 640 mseconds.

A 10-ml sample of fermentation broth was centrifuged (10 minutes, 13,000×*g*, room temperature). The mycelium was extracted with MeOH - acetone (1 : 1), filtered, concentrated to dryness and dissolved in 1 ml MeOH. 10 μl of the samples were injected onto a HPLC column (125×4.6 mm) fitted with a guard column (20×4.6 mm), both of which were packed with 5-μm Nucleosil-100 C-18 (Maisch). Samples were analysed by linear gradient elution using 0.1% *ortho*-phosphoric acid as solvent A and acetonitrile as solvent B, at a flow rate of 2 ml/minute. The gradient was from 0% to 100% solvent B in 15 minutes with a 1-minute hold at 100% solvent B, followed by a 5-minute post-time at initial conditions.

Biological Assays

An agar plate diffusion assay was used to determine the antimicrobial spectrum of the metabolites of strain LU 9575. 10 μl of the samples were applied to filter disks (6 mm diameter). The test plates were incubated for 24~48 hours at a temperature permitting optimal growth of the test

strains.

The inhibitory activities of aspochalamins and aspochalasins on the growth of tumor cells were tested according to NCI guidelines[10] with human cell lines from gastric adenocarcinoma (HMO2), breast carcinoma (MCF7) and hepatocellular carcinoma (HepG2 and Huh7). Huh7 cells express p53 with increased half life as a result of a point mutation at codon 220 and are remarkably insensitive to anticancer drugs[11,12]. Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were exposed to the test compounds for 48 hours.

Results

Taxonomy

The host organism, a woodlouse, collected near Königsheide, Germany, belongs to the family *Trichoniscidae*, which is widespread in the northern hemisphere[13].

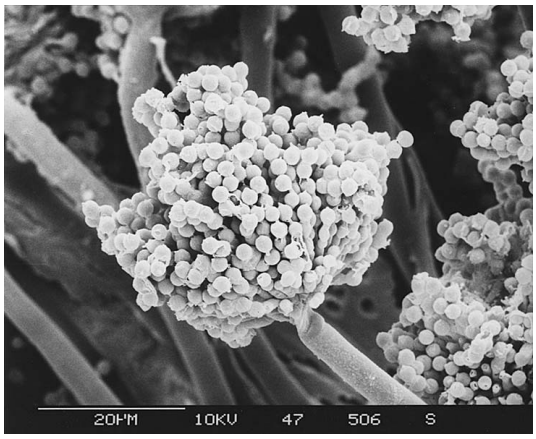
The endosymbiotic strain LU 9575 was characterised by its morphological attributes[9] as a strain of *Aspergillus niveus* Blochwitz. On malt-extract agar conidia were white, colonies reverse uncolored and with dull yellow to brown shades. Conidial heads were columnar and white, stipes were 300 to 600 μm×4 to 7 μm, vesicels were hemispherical 10 to 15 μm in diameter, and conidia were 3.5 μm in diameter, finely roughened and globose. A photomicrograph of the conidial heads is shown in Fig. 1.

Fermentation, Isolation and Characterisation of the Metabolites

In the course of the stationary cultivation of *A. niveus* LU 9575 maximal growth was achieved after 24 days, yielding a biomass dry weight of 0.5 g/liter. Aspochalamins, aspochalasins and citreoviridins were isolated from the mycelium by acetone-MeOH extraction. The HPLC analysis of the mycelium extract is shown in Fig. 2. The three metabolite families were separated from each other by silica gel column chromatography and purified into single compounds by a succession of column chromatographic steps. Isolation from mycelia from 9 liters stationary cultures resulted in 61 mg of aspochalamin A, 30 mg of aspochalamin B, 45 mg of aspochalamin C, 16 mg of aspochalamin D, 11 mg of aspochalasin Z, 35 mg of aspochalasin D, 18 mg of citreoviridin A/C, and 3 mg of citreoviridin B.

The structures of the novel metabolites aspochalamins A~D (Fig. 3) and aspochalasin Z (Fig. 4) were elucidated as described in the following paper[7]. Citreoviridins were initially identified by our HPLC-DAD database[3] and further distinguished by their molecular formulae as determined by ES-FTICR-MS. Aspochalasin D was identified by NMR studies.

Fig. 1. Scanning electron micrograph of *Aspergillus niveus* LU 9575.



Bar, 20 μm

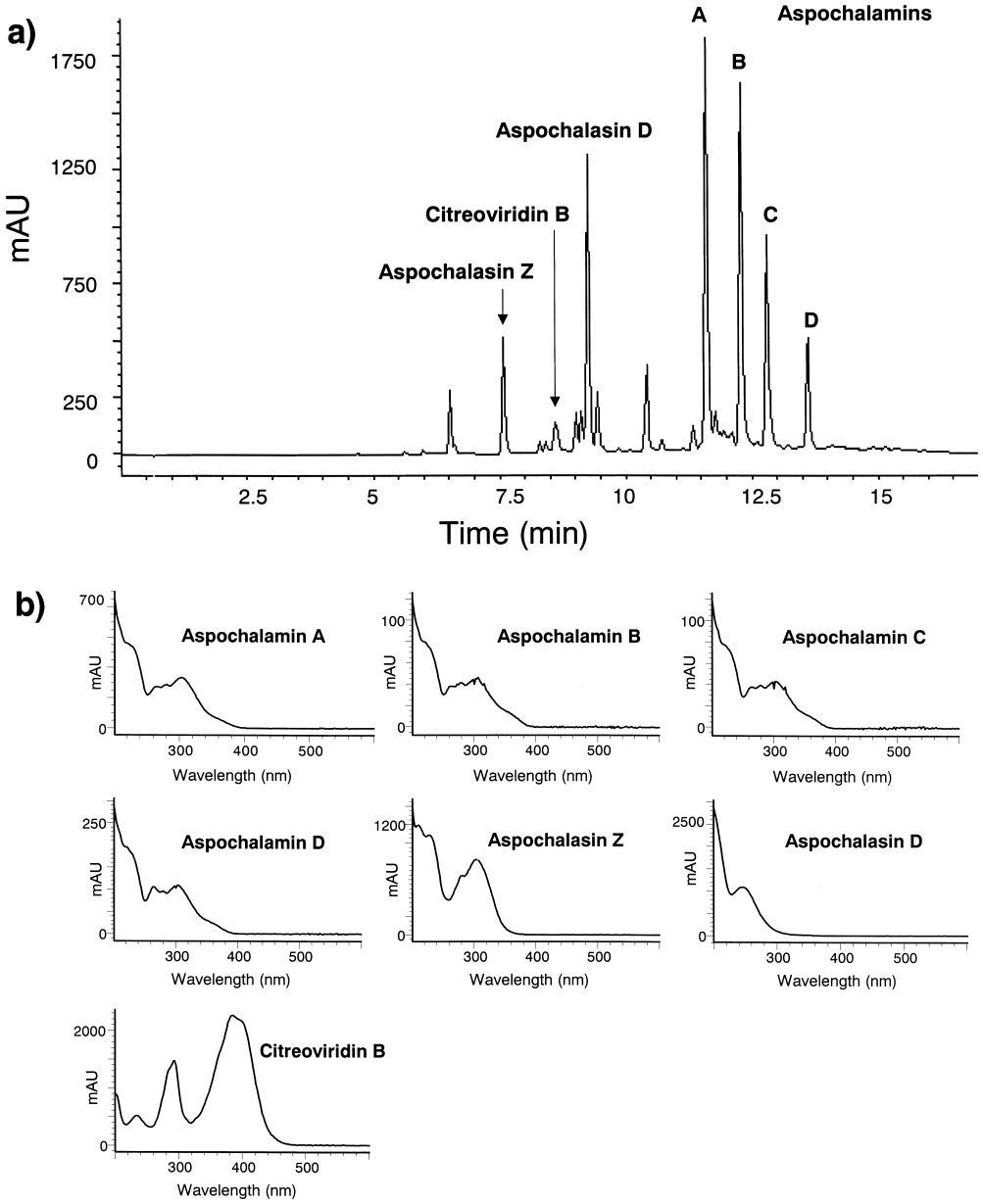
Biological Properties

The antimicrobial spectra of aspochalamins A~D and aspochalasins D and Z were determined by agar plate diffusion assays. Aspochalamins showed a weak antibiotic activity towards Gram-positive bacteria (Table 1). Aspochalamin A was the most active compound within the group of aspochalamins. Gram-negative bacteria (*Agrobacterium tumefaciens* DSM 30205, *Escherichia coli* K12, *Proteus mirabilis* ATCC 35501, *Pseudomonas fluorescens* DSM 50090) were not sensitive to aspochalamins. Fungi (*Sacharomyces cerevisiae* ATCC 9080, *Candida albicans* CBS 12754, *Botrytis cinerea* Tü 157, *Mucor hiemalis* Tü 179/180, *Paecilomyces variotii* Tü 137, *Aspergillus viridi nutans* CBS 12756, *Penicillium notatum* Tü 136) were not inhibited by aspochalamins.

Aspochalasin Z did not exhibit antimicrobial activity towards these test organisms, whereas aspochalasin D was active against several Gram-positive bacteria (Table 1).

The cytostatic effects of aspochalamins A~D and aspochalasin Z were tested in different tumor cell lines. Aspochalamins B and C showed a moderate cytostatic effect in all cell lines tested (GI_{50}: 2.8~7.0 μg/ml).

Fig. 2. HPLC analysis of the mycelium extract from *Aspergillus niveus* LU 9575 monitored at 280 nm (a), and UV-visible spectra from aspochalamins A~D, aspochalasin Z, aspochalasin D and citreoviridin B (b).

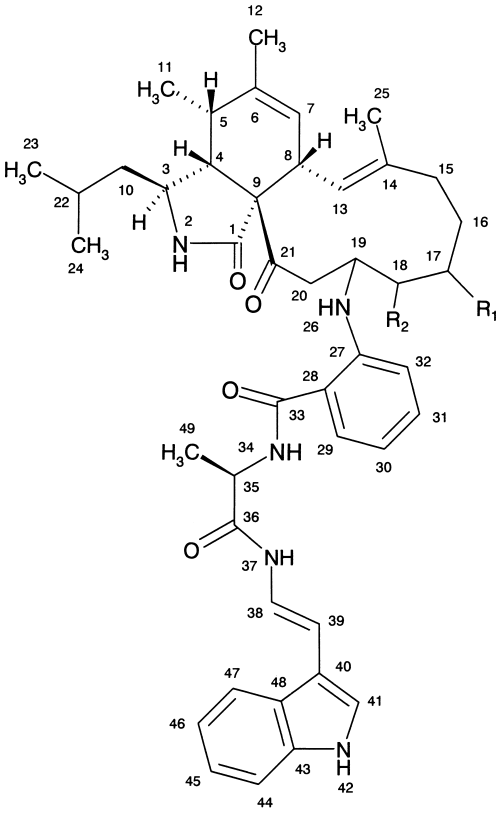


Aspochalamin A displayed a moderate inhibitory potency on the growth of HM02 and MCF7 cells; this effect was less pronounced on HepG2 and Huh7 cells (Table 2). Aspochalasin Z showed a weak cytostatic effect in HepG2, MCF7, and HM02 cells (14~44% growth inhibition at 10 μg/ml), whereas aspochalamin D showed no activity up to 10 μg/ml (data not shown).

Discussion

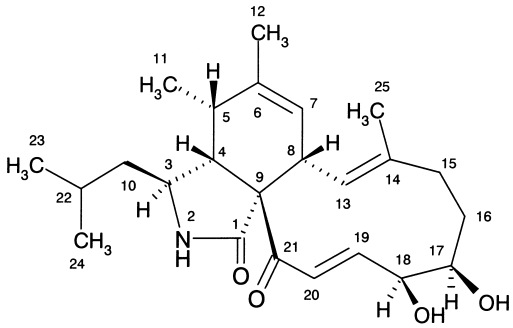
Aspochalasins belong to the cytochalasans, a family of mycotoxins with interesting biological properties[14~18]. In the seventies aspochalasins A, B and the stereoisomers C and D were originally isolated from *Aspergillus microcysticus*[4,5] and since then the aspochalasin family accreted constantly over the years[19~24]. In the course of our screening for bioactive compounds produced by endosymbiotic microorganisms, we isolated the previously

Fig. 3. Structures of aspochalamins A~D.

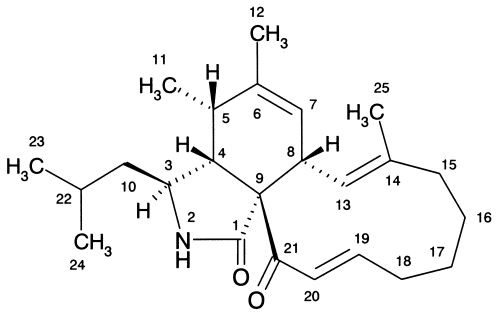


Aspochalamin A: $R^1=R^2=OH$
Aspochalamin B: $R^1=R^2=OH$
Aspochalamin C: $R^1=H$, $R^2=OH$
Aspochalamin D: $R^1=R^2=H$

Fig. 4. Structures of aspochalasins D (top) and Z (bottom).



Aspochalasin D



Aspochalasin Z

unknown aspochalasin Z, as well as aspochalamins A~D, a new family of compounds composed of an aspochalasin skeleton connected to a tripeptidic sequence.

Due to the absence of any oxygen functionalities in positions C-17 to C-20 aspochalasin Z appears to be the 'basic version' of all aspochalasin compounds discovered to date. In batch fermentations of *A. niveus* using stirred tank fermenters with constant aeration rates, only the formation of aspochalasins B and D was observed (data not shown)– production of aspochalasin Z and the aspochalamins occurred exclusively in stationary cultures. Thus, the production of those compounds may be related to the intensity of aeration of the production culture.

Comparison of aspochalasin Z with aspochalasin D (Fig. 4) raises the question whether the C-17 to C-20 hydroxy functions are required for antibacterial activity[25]. Absence of hydroxy groups in positions C-17 and C-18 may also be the explanation for the poor antibacterial effects of aspochalamin D. Unfortunately there are no data published on the antibacterial activities of aspochalasin E[19], which posesses hydroxy groups at C-17, C-18, and C-19.

TOMIKAWA *et al.*[26] suggested that the carbonyl conjugated olefin at C-19 contributed to the cytotoxicity of the aspochalasins. Although aspochalasin Z has this structural feature, this molecule was only poorly cytotoxic. Amongst the aspochalamins, which lack a C-19 double bond, cytotoxicities vary over a broad range; thus, additional structural elements may be responsible for cytotoxic activity. To elucidate that question, detailed structure-activity relationships for the aspochalasins and aspochalamins need to be established.

Citreoviridins are neurotoxic mycotoxins, produced by several *Aspergillus* and *Penicillium* strains[6] and can cause serious poisoning after consumption of contaminated

Table 1. Antibacterial spectrum of aspochalamins and aspochalasin D determined by the agar plate diffusion assay at a concentration of 1 mg/ml.

	Aspochalamin				Aspochalasin
Test organism	A	B	C	D	D
Arthrobacter globiformis DSM 20124	–	–	10	–	9
Arthrobacter aurescens DSM 20116	–	–	–	–	15
Arthrobacter oxydans DSM 6612	–	–	–	–	10
Arthrobacter pascens DSM 20545	8	–	–	–	16
Bacillus subtilis DSM 10	–	–	–	–	13
Brevibacillus brevis DSM 30	16	–	–	–	–
Rhodococcus erythropolis DSM 1069	9	8	7	8	14
Staphylococcus aureus DSM 20231	–	–	–	–	10

Inhibition zones in mm.

Table 2. Activities (μg/ml) of aspochalamins A~C against selected human tumor cell lines.

	GI_{50}				TGI			
Aspochalamin	HM02	MCF7	HepG2	Huh7	HM02	MCF7	HepG2	Huh7
A	2.8	3.1	> 10[a]	> 10[b]	7.2	> 10[d]	> 10	> 10
B	5.3	2.8	6.4	6.5	7.4	9.7	10.0	> 10[f]
C	6.2	6.1	7.0	4.9	> 10[c]	> 10[e]	10.0	9.8

GI_{50}: 50% growth inhibition; TGI: 100% growth inhibition

[a] 29 % inhibition at 10 μg/ml [d] 74 % inhibition at 10 μg/ml

[b] 24 % inhibition at 10 μg/ml [e] 75 % inhibition at 10 μg/ml

[c] 82 % inhibition at 10 μg/ml [f] 78 % inhibition at 10 μg/ml

cereals such as rice[27]. At present we cannot evaluate if aspochalasins, aspochalamins and citreoviridins play a role in the symbiotic relationship between woodlice and their endosymbiotic fungi. *A. niveus* strains, basically know as producers of nivefuranone[28] and citrinin[29,30] have so far not appeared in any symbiotic context and an ecological function, *e.g.* as defense substance like the pederines[31], seems to be speculative.

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