Biologically Active Secondary Metabolites from the Ascomycete A111-95

1. Production, Isolation and Biological Activities

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Eight secondary metabolites were isolated from submerged cultures of the ascomycete A111-95 during a search for new nematicidal metabolites. (−)-Galiellalactone (**7**) and compound **2** are metabolites previously obtained from cultures of *Galiella rufa* while the compounds **1**, **3**, **4**, **5**, **6** and **8** (**3** and **4** were obtained as an unseparable mixture), were isolated as natural products for the first time. Compound **2**, pregaliellalactone (**5**) and the mixture of **3** and **4** showed nematicidal activities towards *Caenorhabditis elegans* and *Meloidogyne incognita*. All compounds showed moderate or weak cytotoxic activities.

During our ongoing screening of asco- and basidiomycetes for nematicidal natural compounds, extracts of the ascomycete strain A111-95 showed activity towards the plant pathogenic nematode *Meloidogyne incognita* and towards the saprophytic *Caenorhabditis elegans*. In this paper the taxonomy of the producing fungus, the fermentation, isolation and biological activities of the active compounds are described, as well as the isolation and biological characterisation of four metabolites lacking nematicidal activity.

Material and Methods

General

Preparative HPLC was performed with a Jasco PU-980 equipped with a Jasco MD910 diode array detector. Analytical HPLC was carried out on a Hewlett Packard HP 1090 equipped with a diode array detector.

Producing Organism

The ascomycete A111-95 was collected from dead wood in Chile in 1995. Mycelial cultures were derived from ascospores. For maintenance on agar slants and submerged cultivation, A111-95 is grown on YMG medium composed of (g/liter): yeast extract 4, malt extract 10, glucose 10, for solid media agar 15 was added. The pH was adjusted to 5.5 prior to autoclaving. The strain is deposited in the culture collection of the LB Biotechnology, University of Kaiserslautern. The collected fruiting bodies had morphological features similar to those of *Urnula craterium*.

Fermentation

Fermentations were carried out in a 20-liter fermenter (Biolafitte C6) containing 20 liters YMG medium with aeration (3.3 liters air/minute) and agitation (120 rpm) at 22°C. As inoculum, a well grown culture (200 ml in a 500 ml Erlenmeyer flask incubated on a rotary shaker at 22°C) in the same medium was used. The production of the nematicidal compounds was followed using the microtiter plate assay. Aliquots (100 ml) of the culture filtrate were extracted with ethyl acetate. The extracts were dried with Na_2SO_4. After evaporation of the solvent the samples were dissolved in 1 ml methanol. These culture filtrate extracts were used in the microtiter plate

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assay.

Isolation

After the complete consumption of the carbon source (glucose), the fermentation was stopped. The culture fluid was separated from the mycelium by filtration. Mycelia containing no active metabolites were discarded. The culture filtrate (60 liters) was extracted with 30 liters ethyl acetate. The organic phase was reduced to an oily extract. This crude extract (15 g) was subjected to a flash chromatography on silica gel (Merck 60; column size 55×450 mm) and stepwise elution with cyclohexane - ethyl acetate 9 : 1 and 8 : 2. An oily intermediate product I (10 g) was obtained at cyclohexane - ethyl acetate 80 : 20. Vacuum liquid chromatography on diol material (80×25 mm, Lichroprep® DIOL, 25-4 μm, Merck) with cyclohexane - *tert*. butyl methyl ether 80 : 20 led to 7.7 g of intermediate product II. The pure compounds were obtained by preparative HPLC (Merck LiChrosorb® Diol, 7 μm, column 250×25 mm) using a linear cyclohexane - *tert*. butyl methyl ether gradient. The compounds eluted at the following solvent mixtures: Compound **1**, 6.7 mg, (90 : 10); compound **2**, 5.7 mg (88 : 12); pregaliellalactone (**5**), 732 mg (85 : 15); the 1 : 1 mixture of **3** and **4**, 115 mg (80 : 20); desoxygaliellalactone (**6**), 259 mg (75 : 25); 4-methoxy-6-pentylpyrone (**8**), 822 mg (50 : 50); galiellalactone (**7**), 2.25 g (10 : 90); followed by a crystallization with cyclohexane - 2-propanol, 90 : 10).

Biological Assays

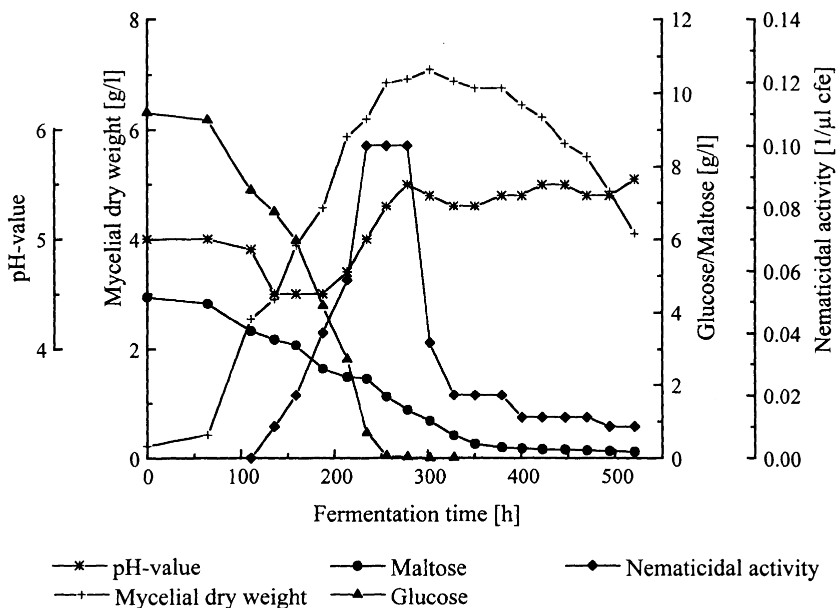
Antimicrobial activity was determined in the agar diffusion assay as described previously[1]. Cytotoxic activities against L1210 cells (ATCC CCL 219), HL 60 cells (ATCC CCL 240), HeLa S3 cells (ATCC CCL 2.2), and COS-7 cells (ATCC CRL 1651) were determined according to ZAPF *et al.*[2]. Inhibition of growth of germinating seeds of *Setaria italica*, *Lepidium sativum* and *Lactuca sativa* was tested as described by ANKE *et al.*[3]. Tests for nematicidal activity were carried out as described by STADLER *et al.*[4] and MAYER *et al.*[5].

Results and Discussion

Taxonomy of the Producing Organism

In its natural habitat on dead wood, the fungus had black, stemless, gelatinous fruiting bodies which were cup-shaped and measured 5~6 cm in diameter. The specimen, unfortunately, could not be identified since the herbarium material had no ascospores. In its morphology the fungus resembled *Urnula craterium* (devil's urn), a wood inhabiting fungus of North America[6]. Due to this morphological similarity, A111-95 belongs in the order *Pezizales* to the family *Sarcoscyphaceae*, tribe *Urnuleae* which harbours among others the genera *Sarcosoma*, *Galiella*, and *Urnula*[7]. This is sustained by the production of the same secondary metabolites by *Galiella rufa* (Schw.)

Fig. 1. Fermentation of A111-95 in YMG medium in a 20-liter fermenter. Nematicidal activity is given as 1/μl culture filtrate extract (cfe).

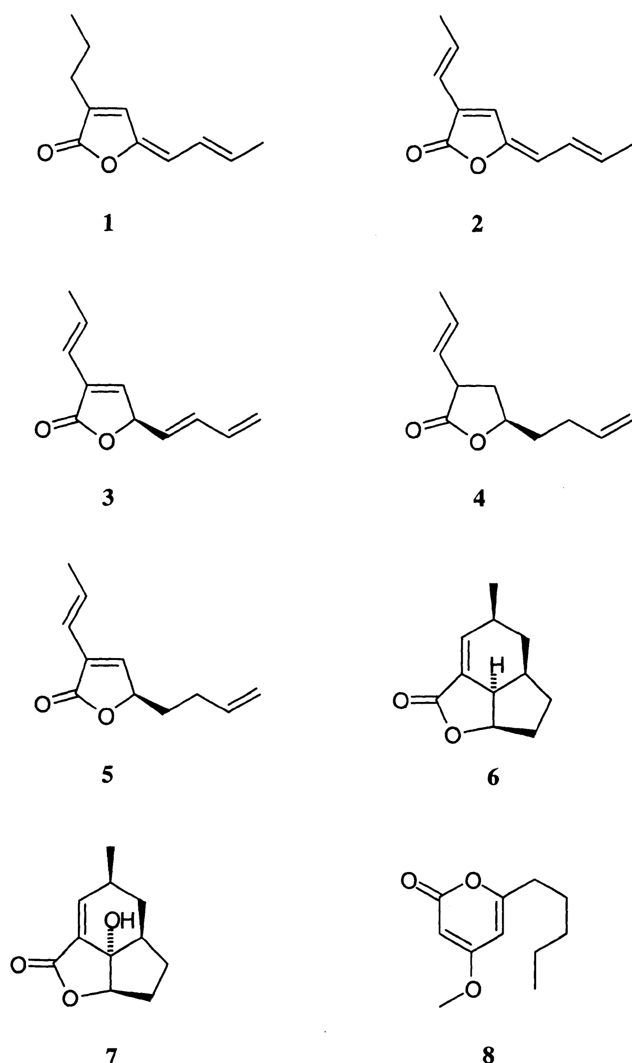


Nannf. & Korf and strain A111-95 (see below).

Fermentation and Isolation of Secondary Metabolites from Strain A111-95

A fermentation diagram of strain A111-95 is shown in Fig. 1. The nematicidal compounds were produced during the growth phase. After a short plateau, the activity dropped very fast. Fortunately the plateau was reached when the glucose in the medium was exhausted and therefore, the cultures were harvested when the glucose had disappeared. Eight metabolites were isolated from the culture filtrate. The structure elucidation of these compounds will be published in a second paper. The structures of the isolated metabolites are shown in Fig. 2. Compounds **1**, **3** and **4** are new natural products. **3** and **4** could in our hands not be separated and were analysed and assayed as a 1 : 1 mixture. Galiellalactone (**7**) and compound **2** have been isolated before from cultures of *Galiella rufa* in the course of a screening for plant growth regulating compounds[8,9]. While the structure of crystalline galiellalactone was obtained by X-ray elucidation, the structure of compound **2** was not elucidated. Galiellalactone (**7**) has also been described as a potent inhibitor of the interleukin-6 signaling from the strain A111-95[10]. Metabolite **5** and desoxygaliellalactone (**6**) have been synthesised as biosynthetic precursors of galiellalactone (**7**)[11,12] while the pyrone **8** has been synthesized by DE MARCH[13].

Fig. 2. Structures of the metabolites isolated from cultures of strain A111-95.



Biological Properties of the Isolated Metabolites

All compounds were tested for nematicidal (Table 1), antimicrobial (Table 2), cytotoxic (Table 3) and phytotoxic activities. The mixture of compounds **3** and **4** showed the highest nematicidal activity, with a LD_{90} value of 60 μg/ml against both *M. incognita* and *C. elegans*. Compound **2** and pregaliellalactone (**5**) showed only weak effects while the remaining compounds were inactive (Table 1). All compounds were only weakly antimicrobial, as can be seen from Table 2, and pregaliellalactone (**5**) had no activity. Table 3 shows that galiellalactone (**7**), in addition to the already reported activities, is cytotoxic towards all tested cell lines. The activity towards suspension cell lines was approximately 10 times higher compared to that towards monolayer cell lines. Compounds **1**, **2**, **3/4**, **5**, and **6** showed

Table 1. Nematicidal activity of compounds **2**, **3/4** and **5** in the microtiter plate assay against *Meloidogyne incognita* and *Caenorhabditis elegans*.

Compound	Nematicidal activity [μg/ml]			
	M. incognita		*C. elegans*	
	LD_{90}	LD_{50}	LD_{90}	LD_{50}
2	125	75-100	>200	150
3/4	60	25-40	60	25-40
5	125	75-100	150	100

Table 2. Antimicrobial activities of compounds **1**~**8** (100 μg were tested on discs 6 mm diameter).
Compound **5** was inactive.

Test organism	Inhibition zone (mm)					
	1	**2**	**3/4**	**6**	**7**	**8**
Bacillus brevis ATCC 9999	–*	–	10	–	12	-
B. subtilis ATCC 6633	–	–	10	–	8	-
Enterobacter dissolvens LMG 2683	–	–	8	–	11	-
Micrococcus luteus ATCC 381	–	–	-	–	8	-
Nematospora coryli ATCC 10647	–	–	8	8	17	-
Penicillium notatum LB Biotechnology	10	8	13	12	-	7
Paecilomyces variotii ETH 4646**	–	–	9	–	–	–
Mucor miehei Tü 284***	–	–	8	10	–	–

- -: no inhibition
- **: Strain collection Eidgenössische Technische Hochschule Zürich, Switzerland
- ***: Strain collection University Tübingen

Table 3. Cytotoxic activities of compounds **1**~**8** towards different mammalian cell lines.

Cell line	IC$_{50}$ [μg/ml]						
	1	**2**	**3/4**	**5**	**6**	**7**	**8**
HL-60	5-10	10-25	5-10	25-50	10-25	1-2	100
L1210	5-10	5-10	2.5-5	25-50	5-10	1-2	100
HeLa S3	>100	>100	50-100	>100	50-100	10-17.5	>100
COS7	>100	>100	>100	>100	50-100	15-25	>100

cytotoxicity towards suspension cell lines only, while the pyrone **8** was almost devoid of cytotoxic activity. An inhibition of germination of *Lepidium sativum*, *Lactuca sativa*, and *Setaria italica* was observed at 600 μg/ml for compounds **5**, **6**, **7**, and **8**, while compounds **1**, **2**, and **3/4** had no phytotoxic effects up to 300 μg/ml, However, a slight reduction of the root growth was detected for compounds **6**, **7**, and **8** (IC$_{50}$ between 65 and 300 μg/ml).

Identification of Pregaliellalactone and Ddesoxygaliellalactone in Cultures of *Galiella rufa*

A reinvestigation of the metabolites in cultures of *G. rufa* A 75-86, the strain from which galiellactone and compound **2** were originally isolated[8,9], revealed the presence of compounds **3/4**, pregaliellalactone (**5**), and desoxygaliellalactone (**6**) in addition to galiellalactone (**7**) in extracts of the culture filtrate. The growth conditions were the same as for strain A111-95.

Chemotaxononic Value of the Compounds 1~7

The natural habitat on dead wood, the morphological similarities of the fruit bodies of strain A111-95 with *Urnula craterium*, and the production of pregaliellalactone (**5**), desoxygaliellalactone (**6**) and galiellalactone (**7**), compounds also produced by in submerged cultures of *Galiella rufa*, support the taxonomic relationship between these fungi. Compounds **5**, **6**, and **7** fit in a

biogenetic scheme proposed before[12]. In this scheme pregaliellalactone is transformed in an intramolecular Diels-Alder reaction to desoxygaliellalactone which subsequently is hydroxylated to galiellalactone. Intramolecular Diels-Alder reactions in fungal biosynthetic pathways are not rare[14], though the only known enzyme up to now is the solanapyrone synthase from *Alternaria solani*[15]. Whether compounds **1**, **2** and **3**/**4** also are part of the biogenetic pathway leading to **7**, or constitute side products, is at present not clear. The pyrone **8**, being a pentaketide, is not biogenetically related to the other metabolites isolated from A111-95 which are of hexaketide origin. Additional fungal strains belonging to the tribe *Urnuleae* are currently being investigated for the production of galiellalactone (**7**) and its biogenetic precursors.

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