LACHNUMON AND LACHNUMOL A, NEW METABOLITES WITH NEMATICIDAL AND ANTIMICROBIAL ACTIVITIES FROM THE ASCOMYCETE *Lachnum papyraceum* (KARST.) KARST.

I. PRODUCING ORGANISM, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITIES

MARC STADLER and HEIDRUN ANKE*

Universität Kaiserslautern, Lehrbereich Biotechnologie,
D-6750 Kaiserslautern, Fed. Rep. Germany

WOLF-RÜDIGER ARENDHOLZ

Universität Kaiserslautern, Lehrbereich Systematische Botanik,
D-6750 Kaiserslautern, Fed. Rep. Germany

FRITZ HANSSKE and UWE ANDERS

Boehringer Mannheim AG,
Sandhofer Str.,
D-6800 Mannheim 31, Fed. Rep. Germany

OLOV STERNER and KARL-ERIK BERGQUIST

Department of Organic Chemistry 2, University of Lund,
S-22100 Lund, Sweden

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Several chlorinated metabolites with nematicidal, antimicrobial, and cytotoxic activities were isolated from submerged cultures of the ascomycete *Lachnum papyraceum*. Three compounds were identified as (+)-mycorrhizin A (**3**), (+)-chloromycorrhizin A (**4**) and (+)-dechloromycorrhizin A (**5**). The occurrence of **5** as a natural product is new. Two compounds, lachnumon (**1**) and lachnumol A (**2**), were found to be new fungal metabolites with cytotoxic, nematicidal and antimicrobial activities.

As fungi and nematodes are living in the same environment, *e.g.* soil, decaying wood and plant materials, there has been a long coevolution of both groups which led to nematode-trapping and destroying fungi as well as to nematodes feeding on fungal mycelia. In these interactions fungal secondary metabolites might play an important role. Therefore a screening of fungi for the production of nematicidal compounds was carried out. Extracts of cultures of the ascomycete A 48~88 showed high nematicidal activities towards *Caenorhabditis elegans*. The producing organisms was identified as *Lachnum papyraceum* (Karst.) Karst. (*Hyaloscyphaceae*), a small, wood-inhabiting ascomycete[1,2]. So far no secondary metabolites have been reported from strains of the genus *Lachnum*. Therefore the nematicidal compounds produced by this fungus were isolated and identified. From the culture filtrate five bioactive compounds were obtained. Three were identified as (+)-mycorrhizin A, (+)-chloromycorrhizin A and (+)-dechloromycorrhizin A[3,4]. The first two compounds have been previously isolated from a mycorrhizal fungus of *Monotropa hypopitys*, dechloromycorrhizin had been obtained during the total synthesis of mycorrhizin A[4]. *Gilmaniella humicola*

has been reported to produce mycorrhizin A and the structurally related mikrolins[5].

In addition to the mycorrhizins, the fungus produced two new compounds which were named lachnumon (**1**) and lachnumol A (**2**). In the following we describe the fermentation of *Lachnum papyraceum*, the isolation of the bioactive metabolites from the culture broth and the biological activities of the metabolites. The elucidation of the structures of lachnumon and lachnumol A will be reported in a second paper[6]. A preliminary report of parts of the results had been presented at the IUPAC Symposium on Natural Products in Strasbourg, 1992[7].

Materials and Methods

General

Materials used for preparative HPLC were obtained from Merck Darmstadt: LiChroSorb Diol (7 μm), LiChroSorb CN (7 μm), LiChroGel PS1 (10 μm). Analytical HPLC was carried out on Hewlett-Packard HP 1090 Type II with a LiChroSpher RP 18 column (10 μm; 125 × 4 mm) and a water - acetonitrile gradient. SIL-A 200 (silicic acid) was obtained from Sigma.

Lachnum papyraceum (Karst.) Karst.

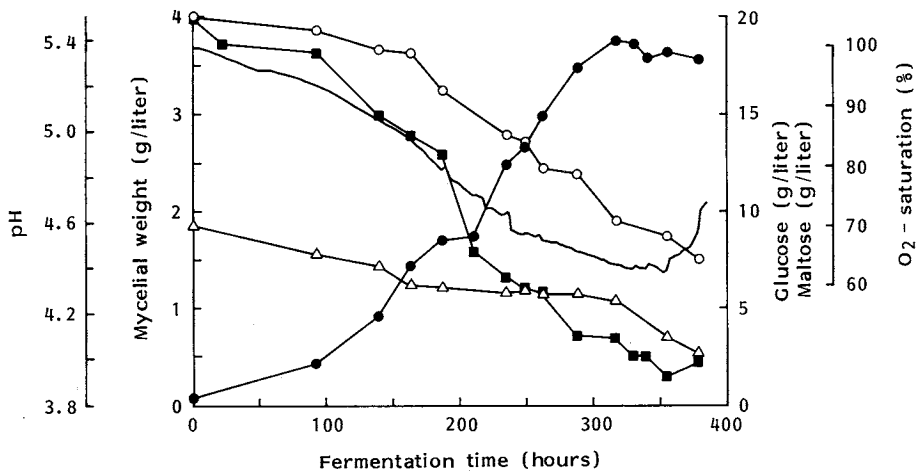
L. papyraceum, strain A 48～88, was collected in 1988 in Hinterstein, Germany. A voucher specimen, which showed the characteristics of the genus and species according to[1,2], and strain A 48～88 (obtained from the ascospores) are deposited in the herbarium and the culture collection of the Lehrbereich Biotechnologie, University of Kaiserslautern.

Fermentation

Strain A 48～88 was maintained and cultivated on MGP medium (maltose 2%, glucose 1%, soypeptone 0.1%, yeast extract 0.1%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%, $CaCl_2 \cdot 2H_2O$ 10 mM, $FeCl_3$ 6 mM, $ZnSO_4 \cdot 7H_2O$ 6 μM). Fermentations were carried out in a 20-liter fermentor (Brain Biostat U) at 24°C with an aeration rate of 3.2 liters/minute and agitation (140 rpm). Oxygen saturation of the culture broth was measured using a Brain Oxygen electrode. Aliquots of the culture fluid (100 ml) were extracted twice with ethyl acetate. The combined extracts were dried with Na_2SO_4. After evaporation of the solvent *in vacuo* (40°C), the oily residue was dissolved in 2 ml of methanol. These extracts were used for determination of **1**～**5** by analytical HPLC (integration of peaks with external and internal standards of the pure

Fig. 1. Fermentation of *Lachnum papyraceum* in 20 liters scale (MGP medium, growth parameters).

● Mycelial dry weight, ■ pH value, —— oxygen saturation of culture broth, △ glucose, ○ maltose.



compounds). Nematicidal activity was followed using *Caenorhabditis elegans* as test organism. Antimicrobial activities were assayed in the agar diffusion assay with *Bacillus brevis* and *Nematospora coryli* as test organisms.

Biological Assays

Antimicrobial activity was determined in the serial dilution assay and the plate diffusion assay as described previously[8,9]. Cytotoxic activities and inhibition of macromolecular syntheses in L1210 cells were determined according to ANKE and STERNER[10].

Nematicidal activities were determined in a microwell plate assay with the free-living nematode *Caenorhabditis elegans*. Worms were cultivated on agar slants as described previously[11]. For the assay a suspension of adult worms and L_4 stages (over 90%) from a five days old culture was diluted with M9 buffer to about 100 nematodes/ml. 330 μl of this suspension were incubated with the compound to be tested in 24 well plates at 18°C in the dark. As a standard, ivermectin was used. Nematicidal activity was recorded after 18 hours.

Results and Discussion

Fermentation of *Lachnum papyraceum* and Isolation of the Metabolites

A typical fermentation diagram is depicted in Fig. 1. The fermentation was terminated after 18 days when the biological activities of the culture fluid (Fig. 2A) decreased. At this time neither glucose nor maltose were used up, but biomass production had ceased, and consequently the O_2 saturation in the culture broth went up. As shown in Fig. 2B, the production of bioactive metabolites started during the trophophase and parallelled mycelial growth. Analytical HPLC revealed that the content of

Fig. 2. Biological activities of the culture filtrate extracts and time course of the production of compounds **1**~**5** during fermentation of *Lachnum papyraceum*.

(A) ▼ Nematicidal activity towards *Caenorhabditis elegans*, ▲ antibacterial activity towards *Bacillus brevis*, □ antifungal activity towards *Nematospora coryli*; (B) ○ lachnumon, △ lachnumol A, ▽ mycorrhizin A, ■ chloromycorrhizin A, ● dechloromycorrhizin A.

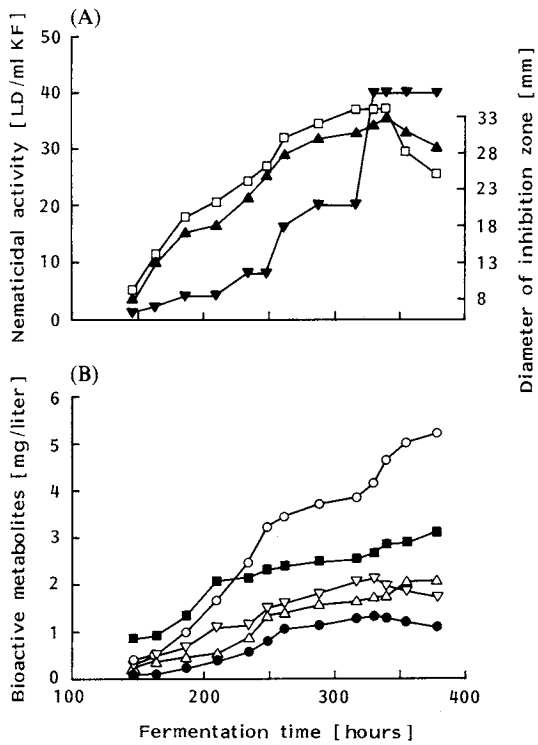


Fig. 3. HPLC profile of a culture fluid extract from *L. papyraceum*.

1=Lachnumon, **2**=lachnumol A, **3**=(+)-mycorrhizin A, **4**=(+)-chloromycorrhizin A, **5**=(+)-dechloromycorrhizin A.

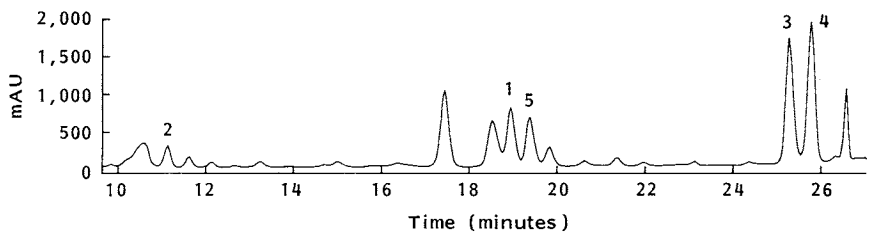
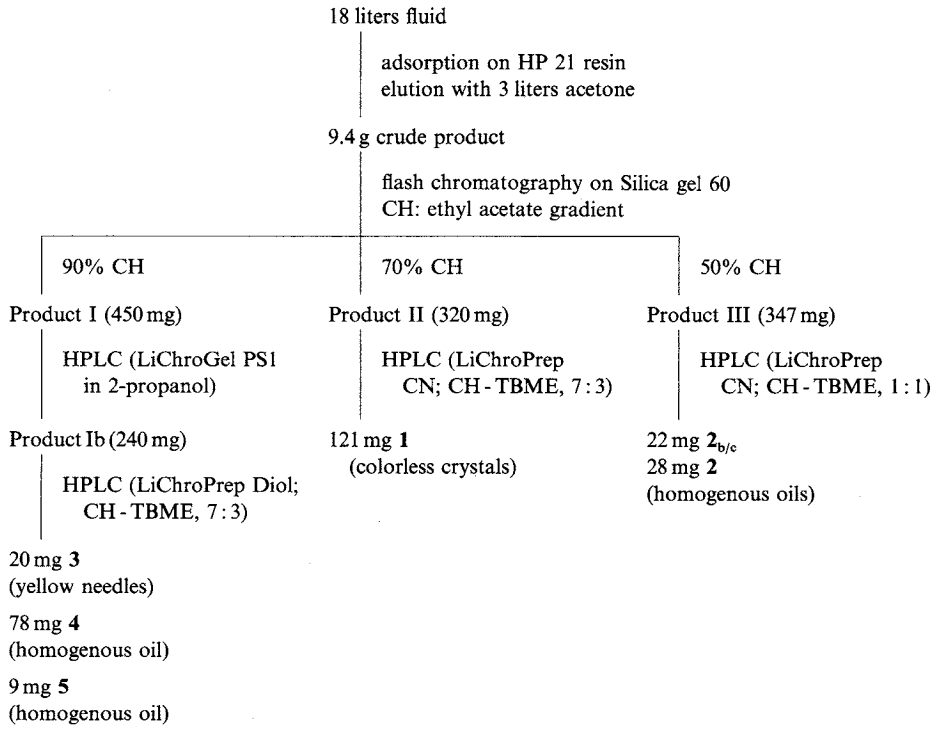


Fig. 4. Isolation of compounds **1**~**5** from the culture filtrate of *Lachnum papyraceum*.

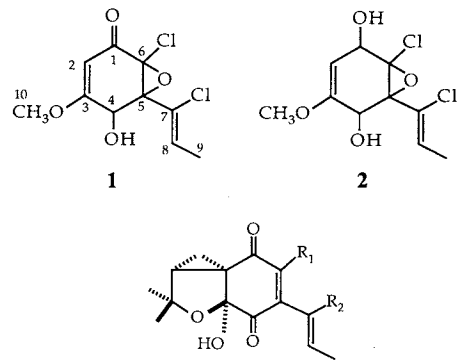


Abbreviations: CH = cyclohexane; TBME = *tert*-butylmethylether.

compounds **1**~**5** steadily increased, but during the last days the content of mycorrhizin A and dechloromycorrhizin A decreased whereas the chloromycorrhizin A content still increased, which may be due to chlorination of **5**, respectively **3** to **4**. Compounds **1**, **2**, lachnumol B/C $\mathbf{2}_{b/c}$ and **5** were detectable after 8 days of fermentation. The isolation of other metabolites, detected by HPLC (see Fig. 3), has not yet been achieved, due to their instability.

The isolation of compounds **1**~**5** from the culture broth is shown in Fig. 4. Mycelia containing no active compounds were discarded. The separation of the mixture composed of $\mathbf{2}_{b/c}$ is currently in progress. The structures are given in Fig. 5.

Fig. 5. Structures of compounds **1**~**5**.



1 = Lachnumon, **2** = lachnumol A, **3** = (+)-mycorrhizin A, **4** = (+)-chloromycorrhizin A, **5** = (+)-dechloromycorrhizin A.

Biological Properties

Antifungal activities of mycorrhizin A and chloromycorrhizin A have been reported previously[3]. All compounds exhibited nematicidal activity towards *Caenorhabditis elegans*, with mycorrhizin A being the most active one. Lachnumon showed only

weak activity as can be seen in Table 1.

The antimicrobial effects are listed in Tables 2 and 3. Whereas **1** and **2** showed rather weak antimicrobial activities, the mycorrhizins **3**~**5** were highly active towards yeasts and bacteria. Similar results were obtained in the plate diffusion assay with filamentous fungi (Table 3). **1**, **2** and $\mathbf{2}_{b/c}$ turned reddish brown after 15 minutes on the paper discs, indicating an oxidation process, which may be one of the reasons for the weak biological activities observed. These compounds are not only sensitive to oxygen, but also unstable in aqueous or methanolic solutions.

As shown in Table 4, rather high cytotoxic activities were observed for **3**~**5**, again, **1** and **2** had weaker effects. Compounds **3**~**5** also strongly

Table 1. Nematicidal activities of **1**~**5** towards *Caenorhabditis elegans*.

Compound	ND_{90} (μg/ml)
Lachnumon (**1**)	25~50
Lachnumol A (**2**)	5~10
Mycorrhizin A (**3**)	1~ 2
Chloromycorrhizin A (**4**)	5
Dechloromycorrhizin A (**5**)	5
Lachnumol B/C ($\mathbf{2}_{b/c}$)	5~10
Ivermectin	0.1

ND_{90}: concentrations (μg/ml) causing over 90% immotility of the worms after 18 hours.

Table 2. Antimicrobial activities of **1**~**5** in the serial dilution assay.

Organism	MIC (μg/ml)					
	1	**2**	**3**	**4**	**5**	$\mathbf{2}_{b/c}$
Bacteria:						
Acinetobacter calcoaceticus	100	>100	25	25	25	100
Bacillus brevis	50	25	1	5	10	100
Bacillus subtilis	50	10	1	2	5	100
Staphylococcus aureus	25	25	2	2	2	25
Yeasts:						
Candida albicans	100	100	10	10	25	100
Nematospora coryli	25	100	1	2	2	100

Table 3. Antifungal activities of **1**~**5** in the plate diffusion assay.

Organism	Diameter of inhibition zone (mm)					
	1	**2**	**3**	**4**	**5**	$\mathbf{2}_{b/c}$
Fusarium oxysporum	11	—*	24	16	14	—
Mucor miehei	—	—	27	15	12	—
Penicillium notatum	15	—	20	12	10	—
Paecilomyces variotii	—	—	19	11	13	—

Concentrations tested: 100 μg/paper disc (i.d. 6 mm).
* —: no inhibition zone.

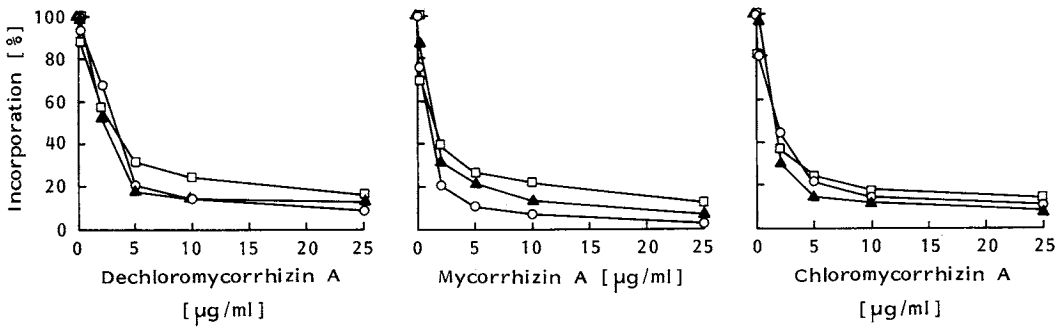
Table 4. Cytotoxic activities of **1**~**5** towards different mammalian cell lines.

Cell line	Concentrations causing total lysis after 24 hours (μg/ml)					
	1	**2**	**3**	**4**	**5**	$\mathbf{2}_{b/c}$
L1210	100	10	0.1	1	1	10
BHK 21	100	100	1.0	1	n.d.*	100
HeLa S3	50	10	0.5	1	1	n.d.
KB	100	25	0.5	1	1	n.d.

* n.d.: not determined.

Fig. 6. Effects of compounds **3**~**5** on the incorporation of labeled precursors into macromolecules in L1210 cells.

○ Incorporation of [^{14}C]thymidine, control (100%)=4,100 cpm; ▲ incorporation of [^{14}C]uridine, control (100%)=16,400 cpm; □ incorporation of [^{14}C]leucine, control (100%)=32,600 cpm.



inhibited the incorporation of precursors into DNA, RNA and protein. At concentrations of 1 μg/ml all three macromolecular biosyntheses were inhibited more than 50% as shown in Fig. 6.

In addition, **3**~**5** readily formed adducts with cysteine in biomimetic experiments[12]. This unspecific mode of action is in agreement with the inhibition of all macromolecular syntheses. **3** and **4** also weakly inhibited the chitin synthase of *Coprinus cinereus*[13], whereas **5** reduced AMV reverse transcriptase activity at 50 μg/ml to 50%[14]. Aggregation of bovine thrombocytes was inhibited by **3**, **4** and **5** at 33 μg/ml[15].

1 and **2** did not form cysteine adducts and no activities in the assays mentioned above were observed. None of the compounds inhibited the respiration of fungi or bacteria or had hemolytic activity at a concentration of 100 μg/ml.

Compounds **3**~**5** differ only in their chlorine substitution patterns. Mycorrhizin A (**3**) showed the highest nematicidal, antimicrobial and cytotoxic activities. Chlorine substitution in the side chain therefore seems to increase biological activity, whereas chlorine substitution within the ring systems weakens it. The occurrence of **5** as a natural metabolite is also interesting for biosynthetical reasons as until now it was not clear at which stage during the biosynthesis the chlorinations are carried out[5]. Now it seems likely that **5** is transformed to **4** *via* **3**.

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