Metabolites with Nematicidal and Antimicrobial Activities from the Ascomycete *Lachnum papyraceum* (Karst.) Karst

V. Production, Isolation and Biological Activities of Bromine-containing Mycorrhizin and Lachnumon Derivatives and Four Additional New Bioactive Metabolites

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Eight novel bioactive metabolites were isolated from submerged cultures of the ascomycete *Lachnum papyraceum* (Karst.) Karst, when $CaBr_2$ was added to the cultures after the onset of secondary metabolism. Four of these metabolites (**16**~**19**) are bromo analogues of mycorrhizin A and lachnumon, while (1′*Z*)-dechloromycorrhizin A (**12**) and the papyracons A (**13**), B (**14**), and C (**15**) are non-halogenated compounds structurally related to the mycorrhizins. All compounds exhibited antimicrobial, cytotoxic, nematicidal and phytotoxic activities. The brominated mycorrhizins and lachnumons were found to be slightly less active than the chlorine-containing compounds. All mycorrhizin derivatives were mutagenic in the Ames test, suggesting DNA-alkylating properties.

During investigations on the secondary metabolism of the ascomycete *Lachnum papyraceum*, several novel chlorinated and brominated isocoumarin derivatives were obtained when the fungus was cultured in $CaBr_2$-containing medium. But no bromo analogues of chlorine-containing lachnumon (**1**) or mycorrhizin A (**3**) were detected, instead the biosynthesis of these compounds was suppressed[1~4]. As previous studies have indicated that the halogenation occurs during the final steps of the mycorrhizin biosynthesis, and that dechloromycorrhizin A (**5**) might be a substrate for halogenation[3], it appeared logical to add bromide at a later stage of the fermentation when the production of **5** already had started.

In this paper we describe the production, isolation and biological activities of four new brominated metabolites and four novel non-halogenated antibiotics. The compounds were obtained from fermentations of *L. papyraceum* when $CaBr_2$ was added to the culture after the onset of secondary metabolism. The structural elucidation of the eight new compounds will be the subject of the two following papers[5,6].

Materials and Methods

General

Materials and methods for fermentation of *L. papyraceum*, detection and isolation of bioactive compounds have been previously reported in papers of this series[1,3].

Biological Assays

Methods for determination of nematicidal, antimicrobial, and cytotoxic activities and the preparation of cysteine adducts have been previously described[3]. The Ames test was carried out as a "pour plate test" without S9 mix[7], using different strains of *Salmonella typhimurium*. Test plates (diameter 5 cm) with 1 ml of top agar on 5 ml of Vogel Bonner medium were used. The experiments were repeated three times with each concentration in triplicate.

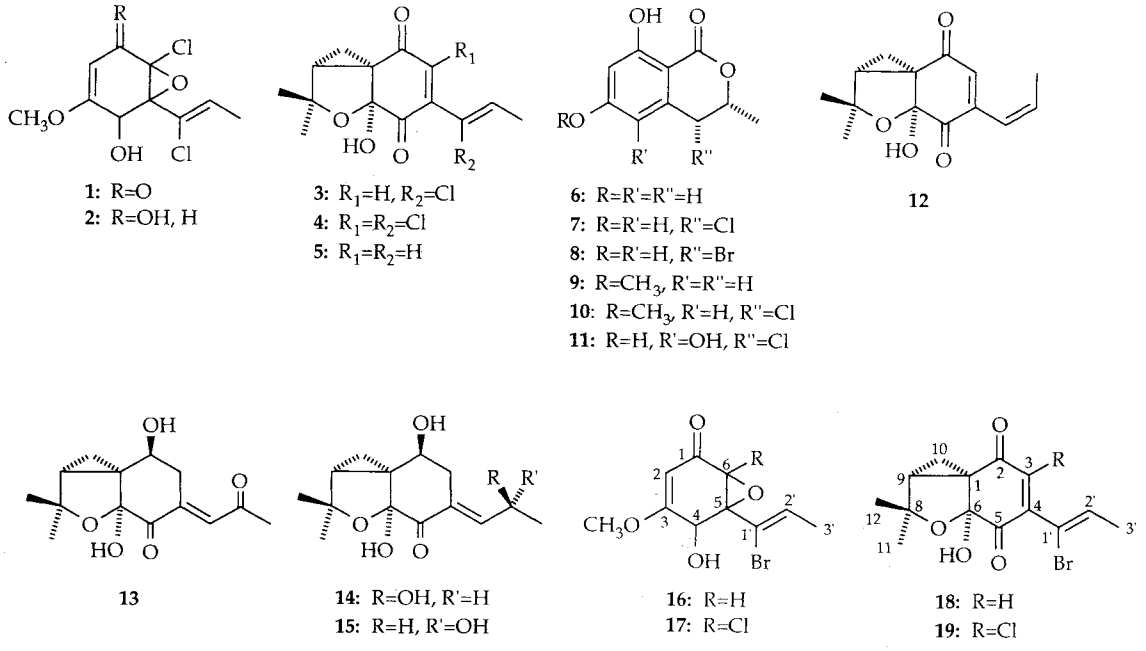
Fermentation of *L. papyraceum*

The fermentation, preparation of extracts and the determination of biological activities were carried out as described previously[3]. The production of secondary metabolites was followed by analytical HPLC[3]. Fermentations were carried out in MGP medium[3], $CaBr_2$ was added after dechloromycorrhizin A (**5**) could be detected in extracts of the culture filtrate.

Isolation of Compounds **12**~**19**

The culture fluid (18 liters) obtained by filtration was applied onto HP 21 resin (1.2 kg), which was eluted with 3 liters of acetone, followed by evaporation of the ace-

Fig. 1. Structures of compounds **1**~**19** produced by *L. papyraceum*.



1: R=O
2: R=OH, H

3: R_1=H, R_2=Cl
4: R_1=R_2=Cl
5: R_1=R_2=H

6: R=R'=R''=H
7: R=R'=H, R''=Cl
8: R=R'=H, R''=Br
9: R=CH_3, R'=R''=H
10: R=CH_3, R'=H, R''=Cl
11: R=H, R'=OH, R''=Cl

12

13

14: R=OH, R'=H
15: R=H, R'=OH

16: R=H
17: R=Cl

18: R=H
19: R=Cl

1=Lachnumon, **2**=lachnumol A, **3**=mycorrhizin A, **4**=chloromycorrhizin A, **5**=(1'*E*)-dechloromycorrhizin A, **6**=6-hydroxymellein, **7**=4-chloro-6-hydroxymellein, **8**=4-bromo-6-hydroxymellein, **9**=6-methoxymellein, **10**=4-chloro-6-methoxymellein, **11**=4-chloro-6,7-dihydroxymellein, **12**=(1'*Z*)-dechloromycorrhizin A, **13**=papyracon A, **14**=papyracon B, **15**=papyracon C, **16**=lachnumon B1, **17**=lachnumon B2, **18**=mycorrhizin B1, **19**=mycorrhizin B2.

tone *in vacuo* and extraction of the remaining aqueous phase with 3 × 500 ml ethyl acetate. The combined ethyl acetate extracts were dried with Na_2SO_4 and evaporated to an oily residue (7.8 g). This crude extract was separated into five intermediate products by flash chromatography on silica gel 60 (column size: 4 cm × 20 cm), using a cyclohexane - ethyl acetate gradient.

Intermediate product 1 (237 mg) was eluted with 1 liter cyclohexane - ethyl acetate (8 : 2). HPLC on LiChroPrep Diol (cyclohexane - *t*-butyl methyl ether; 7 : 3) led to the isolation of 57 mg dechloromycorrhizin A (**5**), 34 mg of (1'*Z*)-dechloromycorrhizin A (**12**), 59 mg of intermediate product 1a, and 54 mg of intermediate product 1b. Each of these intermediate products was subjected to HPLC on LiChroGel PS1 (10 μm) in 2-propanol, yielding 34 mg of mycorrhizin A (**3**) and 9 mg of mycorrhizin B1 (**18**) from intermediate product 1a. From intermediate product 1b, 33 mg of chloromycorrhizin A (**4**) and 14 mg of mycorrhizin B2 (**19**) were obtained.

From 490 mg of intermediate product 2 (obtained by elution with cyclohexane - ethyl acetate; 7 : 3), the isocoumarin derivatives **6** (34 mg), **7** (29 mg), and **8** (53 mg) were obtained by a similar isolation procedure as described previously[1].

The intermediate products 3~5 were eluted subsequently from the column. Elution with 1 liter cyclohexane - ethyl acetate (1 : 1) resulted in intermediate product 3 (360 mg), which was further purified on LiChroPrep Diol with cyclohexane - *t*-butyl methyl ether (1 : 1), yielding 11 mg of lachnumon B1 (**16**) and intermediate product 3a (145 mg). From intermediate product 3a, lachnumon (**1**; 89 mg) and lachnumon B2 (**17**; 11 mg) were isolated by repetitive HPLC on LiChroGel PS1 in 2-propanol.

Intermediate product 4 (240 mg) was obtained at 500 ml cyclohexane - ethyl acetate (1 : 2) and yielded 41 mg of lachnumol A (**1**) and 41 mg of papyracon A (**13**) after HPLC on LiChroSorb CN (cyclohexane - ethyl acetate; 1 : 1).

Intermediate product 5 (140 mg) was eluted with additional 500 ml cyclohexane - ethyl acetate (1 : 2). HPLC on LiChroGel PS1 in 2-propanol yielded a two-component mixture (intermediate product 5a), from which the stereoisomers, papyracons B (**14**; 20 mg) and C (**15**; 25 mg) were separated by HPLC on LiChroSorb Diol (*t*-butyl methyl ether - 2-propanol; 9 : 1).

Results and Discussion

Fermentation of *L. papyraceum*

During the fermentation, dechloromycorrhizin A (**5**) was detected in extracts of the culture broth after ten days and subsequently $CaBr_2$ (50 mM) was added. The addition of $CaBr_2$ had no influence on the production of fungal biomass. The biological activities of extracts were only slightly weaker as compared to extracts from bromide-free MGP medium[3] but much higher as compared to fermentations with $CaBr_2$ added at the beginning[1]. The composition of the extracts, however, changed after the bromide salt had been added. Beside 6-

hydroxymellein (**6**), its 4-chloro- and 4-bromo-derivatives (**7** and **8**), two new peaks corresponding to compounds **18** and **19**, with UV spectra similar to those of compounds **3** and **4**, were detected by HPLC analysis (the spectroscopic data of the new compounds are given in the following paper). In comparison to the chlorinated mycorrhizins, compounds **18** and **19** showed slightly higher retention times on reversed phase materials, suggesting an increased lipophilicity of the new products. This indicated that bromide and not chloride might have been incorporated into dechloromycorrhizin.

HPLC analysis of the intermediate products **3**~**5** revealed the presence of additional metabolites with lachnumon-like UV spectra. In the crude extracts these compounds were hardly detectable, as their concentrations were below 1 mg/liter. They were isolated in small amounts and structural elucidation[5,6] showed that two of these metabolites (**16** and **17**) are bromo analogues of lachnumon. Three metabolites (**13**~**15**) had the same carbon skeleton as the mycorrhizins but were named papyracon A, B, and C, as they differ significantly from the mycorrhizins.

In comparison to the fermentation when $CaBr_2$ was added at the beginning[1], the total amount of secondary metabolites was similar, *e.g.* 535 mg from a 20-liter fermentor in this study and 575 mg in the previous one[1]. However, the yields of halogenated isocoumarin derivatives (**7** and **8**) were considerably lower here (82 mg compared to 476 mg), and there were no traces of compound **9**~**11**. Compound **12** was also present in extracts from bromide-free MGP medium[3] but due to its instability, the isolation and structural elucidation of **12** could not be completed earlier and is therefore reported here and in the following paper.

So far, all brominated fungal metabolites were obtained as the bromo analogues of chlorine-containing metabolites[8~12], and their production in significant amounts under natural conditions does not seem very likely. The ecological importance of chlorinated fungal metabolites was recently examplified by the role that chlorinated anisyl alcohols and aldehydes play during both lignin and forest litter degradation[13].

Biological Activities of Compounds **12**~**19**

The nematicidal effects of **12**~**19** listed in Table 1 show that *Caenorhabditis elegans* is sensitive to all compounds, the new mycorrhizins **12**, **18**, and **19** being the most potent ones. Compared to the chlorinated compounds (**3** and **4**), the brominated analogues were slightly less active. The same is true for lachnumon B2 (**17**) and lachnumon (**1**).

In the agar diffusion assay (Table 2), *Penicillium notatum*, *Paecilomyces variotii* and *Mucor miehei* were inhibited by the new mycorrhizins (**12**, **18**, and **19**). Only *P. notatum* was inhibited by **13**, **16**, and **17**. In the serial dilution assay (Table 3), rather weak antimicrobial activities towards bacteria and yeasts were found for **13**~**17**, whereas the activities of **12**, **18**, and **19** were similar to those of the mycorrhizins **3**~**5**[3]. As shown in Table 4, compounds **12**, **13**, **18**, and **19** showed cytotoxic effects, whereas the activities of **14**~**17** only were weak. In addition, **12**~**19** had weak phytotoxic effects on the growth of *Setaria italica* and *Lepidium sativum* (data not shown). Compounds **12**, **13**, **18**, and **19** reacted with L-cysteine, forming ninhydrin-positive adducts which were devoid of biological activities. The mutagenic ac-

Table 1. Nematicidal activities of compounds **1**, **3**~**5** and **12**~**19** towards *Caenorhabditis elegans*.

Compound	ND_{90} (μg/ml)
1	25~50
3	1~2
4	5
5	5
12	5
13	25
14	100
15	100
16	25
17	50
18	5
19	10
Ivermectin	0.1

ND_{90}: Concentrations causing more than 90% immotility after 18 hours.

Table 2. Antifungal activity of compounds **1**, **3**~**5** and **12**~**19** in the agar diffusion assay after 24 hours with 50 μg/paper disk (6 mm). **14** and **15** were inactive.

Organism	Diameter of inhibition zone (mm)									
	1	**3**	**4**	**5**	**12**	**13**	**16**	**17**	**18**	**19**
Mucor miehei	—	27	12	14	14	—	—	—	20	15
Penicillium notatum	15	20	10	13	13	15	10	14	19	13
Paecilomyces variotii	—	19	13	12	12	—	—	—	21	14

Table 3. Antimicrobial activities of compounds **12**~**19** in the serial dilution assay.

Organism	MIC (μg/ml)							
	12	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Bacteria (Nutrient broth)								
Acinetobacter calcoaceticus	25	50	>100	>100	100	100	25	50
Bacillus brevis	10	50	100	100	100	100	2	5
Bacillus subtilis	10	50	100	100	100	100	2	10
Micrococcus luteus	10	>100	100	100	>100	100	25	25
Yeasts (YMG medium)								
Candida albicans	10	100	>100	>100	50	50	10	10
Nematospora coryli	2	10	50	50	50	100	2	2
Rhodotorula glutinis	25	50	>100	>100	100	100	25	50
Saccharomyces cerevisiae	10	>100	50	25	25	>100	10	25

Table 4. Cytotoxic activities of compounds **3**~**5** and **12**~**19** towards mammalian cell lines.

Cell line	IC_{100} (μg/ml)										
	3	**4**	**5**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
L 1210	0.1	0.5	1.0	2.0	10.0	50.0	50.0	50.0	50.0	2.0	2.0
HL 60	0.5	0.5	0.5	1.0	10.0	25.0	50.0	50.0	50.0	2.0	2.0
HeLa S3	0.5	2.0	2.0	2.0	10.0	100.0	100.0	25.0	10.0	2.0	2.0
BHK 21	1.0	2.0	2.0	2.0	10.0	50.0	50.0	25.0	10.0	2.0	10.0

IC_{100}: Concentration causing total lysis of the cells.

Table 5. Mutagenic effects of compounds **3**~**5**, **12**, **18** and **19** towards different *Salmonella typhimurium* strains without metabolic activation. The response is given as the average number of revertants/plate.

Compound	Concentration (μg/plate)	Number of revertants/plate strain (*S. typhimurium*)			
		TA 97	TA 98	TA 100	TA 102
None		90	19	61	32
3	2	78	13	102	81
4	2	73	30	189	119
5	5	300	21	830	>5,000
12	5	150	17	1,050	3,200
18	5	300	13	870	41
19	5	210	26	260	110
Daunomycin (2 μg/plate)		>5,000	3,500	90	1,800
MES (2 μl/plate)		117	112	>5,000	>5,000

tivities of the mycorrhizins (Table 5) indicate the biomolecule-alkylating potency of these compounds. Strains suitable for the detection of base pair mutations showed a significant response, whereas the "frame shift strains" *S. typhimurium* TA 98 and TA 97 were not or less sensitive. The highest responses were obtained with the two dechloromycorrhizin A isomers (**5** and **12**). Papyracon A (**13**) inhibited growth of all *Salmonella* strains at 25 μg/plate, but was not mutagenic, neither were compounds **1**, **2**, **7**~**11**, **14**~**17**. The influence of microsomal activation remains to be investigated.

Compounds **14**, **15**, and **16** did not inhibit the aggregation of bovine thrombocytes[14] at 132 μg/ml, **12**, **13**, **18**, and **19** were active at 33 μg/ml and lachnumon B2 (**17**) at 66 μg/ml.

The biological activities of (1′*Z*)-dechloromycorrhizin A (**12**) resembled those of its (1′*E*)-isomer **5** in most biological assays. The biological effects of **16** and **17** were similar to those of lachnumon (**1**). Papyracon A (**13**) was more active than its corresponding alcohols (**14** and **15**).

The brominated mycorrhizins showed weaker activities than their chlorinated analogues. During studies on the reactivity of **3** to **18** with thiols, mycorrhizin B1 (**18**) reacted slowly, whereas mycorrhizin A (**3**) lost its activity immediately and was hardly detectable after 5 seconds. Similar results were obtained with chloromycorrhizin A (**4**) and its bromo analogue **19**.

Generally, changes in the biological activities following the substitution of chlorine by bromine are difficult to predict. Brominated pyrrolomycins showed higher antimicrobial effects in comparison to their parent (chlorinated) compounds[15,16] while ten-fold weaker effects were observed in the case of the pyrrolnitrins[17]. Brominated rebeccamycins showed antitumor activities similar to their chloro analogues[18], whereas the bromine-containing duocarmycins showed higher toxicity in mice and were more active towards tumor cells[19].

Mycorrhizins or lachnumons containing two bromine

substitutions were not yet detected and it will be a challenge to find suitable conditions for the production of such compounds as well as to obtain metabolites containing fluorine or iodine, which up to now have never been reported as secondary metabolites in fungi[20,21].

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