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

III. Production of Novel Isocoumarin Derivatives, Isolation, and Biological Activities

MARC STADLER and HEIDRUN ANKE

Universität Kaiserslautern, Lehrbereich Biotechnologie,
Paul-Ehrlich-Str. 23, D-67663 Kaiserslautern, Fed. Rep. Germany

OLOV STERNER

Department of Organic Chemistry 2, University of Lund,
P.O.B. 124, S-22100 Lund, Sweden

(Received for publication May 2, 1994)

During investigations on the influence of $CaBr_2$ on the secondary metabolism of *Lachnum papyraceum*, the production of mycorrhizins and lachnumon type antibiotics was strongly inhibited in bromide-containing culture media. Instead, six isocoumarin derivatives, 6-hydroxymellein (**6**), 4-chloro-6-hydroxymellein (**7**), 4-bromo-6-hydroxymellein (**9**), 6-methoxymellein (**10**), 4-chloro-6-methoxymellein (**11**), and 4-chloro-6,7-dihydroxymellein (**12**) were isolated. Compounds **7**, **9**, **11**, and **12** have never been isolated from natural sources. 6-Hydroxymellein has been isolated previously from many sources including *Gilmaniella humicola* and proposed to be a precursor of mycorrhizin A (see reference 6). In comparison to the mycorrhizins, the isocoumarin derivatives exhibited only weak antimicrobial, cytotoxic, phytotoxic, and nematicidal activities.

Many ascomycetes and basidiomycetes are known to produce chlorine-containing secondary metabolites, most of which are biologically active[1].

During a screening for new nematicidal compounds in cultures of higher fungi, the ascomycete *Lachnum papyraceum* was found to produce several nematicidal metabolites like the mycorrhizins (**3**~**5**), lachnumol A (**2**), and lachnumon (**1**)[2,3]. All of these compounds contain one or two chlorine substitutions. As other fungi had previously been reported to produce the brominated analogues of chlorinated antibiotics[1], the influence of bromide and fluoride on the secondary metabolism of *L. papyraceum* was investigated. No brominated analogues of lachnumol or mycorrhizins were obtained in $CaBr_2$-containing media. In this paper, we describe an interesting shift in the secondary metabolism of this ascomycete in the presence of $CaBr_2$, leading to the isolation and characterization of new bioactive natural products. Their structural elucidation will be published separately in the following paper[4].

Materials and Methods

General

The description of the producing organism, the assays for the determination of the biological activities, the equipment for the detection, isolation, and structure elucidation of the bioactive compounds have been reported previously[2,3]. The plant germination assay was carried out, following the method described by ANKE *et al.*[5]. Columns for both MPLC and HPLC measured 250 × 25 mm.

Fermentation of *L. papyraceum* and Isolation of Bioactive Compounds

MGP medium[2] containing $CaCl_2$ (5 mM) and $FeCl_3$ (6 μM) was the basic culture medium for the fermentation of *L. papyraceum*. In MGP1, the $CaCl_2$ was replaced by $CaBr_2$ (5 mM); in MGP2 the $CaCl_2$ was replaced by $CaBr_2$ (50 mM); in MGP3 $CaBr_2$ (5 mM) was added to MGP; in MGP4 $CaBr_2$ (50 mM) was added; in MGP5 CaF_2 (5 mM) and in MGP6 CaF_2 (50 mM) were added. For preliminary experiments, fermentations were carried out in 2-liter Erlenmeyer flasks containing 1 liter of different MGP media (MGP1~MGP6). The cultures were incubated at 24°C on a rotary shaker (120 rpm). After 18 days, the cultures were harvested and the culture fluids were extracted with ethyl acetate (1 liter). The organic extracts were dried *in vacuo* at 40°C. Aliquots were tested for biological activities and their composition was analysed by the HPLC system used for the detection of mycorrhizins and lachnumons[2].

For production of compounds **6**, **7**, and **9**~**12**, the

† Dedicated to Prof. Dr. HANS ZAEHNER, University of Tübingen, on the occasion of his 65th birthday.

fungus was cultivated in a 20-liter fermentor (Biolafitte C6) at 22°C, using MGP4 medium with 50 mM $CaBr_2$. The fermentation was terminated after 18 days, when the biological activities started to decrease. The mycelium was separated from the fluid and discarded. The culture fluid was applied onto Mitsubishi HP 21 resin and the active compounds were eluted with acetone, followed by evaporation of the acetone *in vacuo* and extraction of the remaining aqueous phase with ethyl acetate. Evaporation of the ethyl acetate *in vacuo* yielded 8.2 g of an oily crude product, which was subjected to MPLC on silica gel 60 (Merck; 25~40 μm). Three intermediate products were obtained by elution with cyclohexane-ethyl acetate. Compounds **6**, **7**, **9**~**12** were isolated from these intermediate products as described in the following:

Intermediate product 1 (26 mg; eluted with 10% ethyl acetate) yielded 8 mg of **10** (Rt 56 minutes) and 5 mg of **11** (Rt 67 minutes) after HPLC on LiChroGel PS1 (10 μm; flow rate 5 ml/minute) in 2-propanol.

Intermediate product 2 (1,370 mg; eluted with 20% ethyl acetate) was further separated by HPLC on LiChroSorb Diol (7 μm; flow rate 5 ml/minute) with a cyclohexane - tert. butylmethylether gradient (60 minutes at 70% cyclohexane; 90 minutes from 30% to 100% tert. butylmethylether). Besides 3 mg of compound **3**, 2 mg of **4** and 16 mg of **5** (eluted with 70% cyclohexane), 45 mg of crude product 2a (eluted with 50% cyclohexane) and 540 mg of crude product 2b (eluted with 30% cyclohexane) were obtained. Crude product 2a yielded 29 mg of **6** (Rt 39 minutes) after HPLC on LiChroGel PS1 in 2-propanol. Crude product 2b yielded 287 mg of **7** (Rt 43 minutes) and 189 mg of **9** (Rt 46 minutes) after repetitive HPLC on LiChroGel PS1 in 2-propanol.

Intermediate product 3 (69 mg; eluted with 50% ethyl acetate) yielding 13 mg of **12**, 14 mg of **1** and 5 mg of **2** after HPLC on LiChroSorb CN (7 μm) by elution with cyclohexane - tert. butylmethylether (1 : 1). In the numbering of the isocoumarins, **8** was omitted, as it stands for a synthetic derivative needed for the elucidation of the structures (see following paper on the structure elucidation). Thus, the natural compounds in this and in the following paper[4] bear the same number.

Results

When $CaCl_2$ was replaced by $CaBr_2$ (MGP1 and MGP2), the cultures showed only sparse mycelial growth and almost no production of secondary metabolites. When bromide or fluoride salts were added in the presence of $CaCl_2$ (MGP3, MGP4, MGP5 and MGP6), the ascomycete showed similar growth like in MGP medium. Mycelial weight, coloration of the culture, time course of the consumption of glucose and pH of filtrate were the same.

In CaF_2 containing media (MGP5 and MGP6), no changes in the composition of the extracts were observed. The addition of 5 mM or 50 mM $CaBr_2$ (MGP3 and MGP4), however, caused some drastic changes in the composition of the extracts, which became evident by HPLC analysis. Fig. 1 shows the HPLC profile of a culture fluid extract from MGP3 with 5 mM $CaBr_2$. Lachnumon (**1**), chloromycorrhizin A (**4**), and dechloromycorrhizin A (**5**) were not detectable, although lachnumol A (**2**) and mycorrhizin A (**3**) were still present in traces. Instead, the production of previously not detected metabolites, which are indicated in Fig. 1 as **6**, **9**, **10**, **11** and **12** was observed. Production of **7** was greatly enhanced. Compound **7** was produced in $CaBr_2$-free medium, the amount however was less than 0.3 mg/liter and not high enough to allow an identification. The UV spectra of compounds **6**, **7**, **9**~**12**

Fig. 1. HPLC profile of a culture fluid extract from *L. papyraceum* grown in MGP3 medium (RP 18; water-methanol).

2=lachnumol A; **3**=mycorrhizin A; **6**: 6-hydroxymellein; **7**=4-chloro-6-hydroxymellein; **9**=4-bromo-6-hydroxymellein;**10**=6-methoxymellein; **11**=4-chloro-6-methoxymellein; **12**= 4-chloro-6,7-dihydroxymellein.

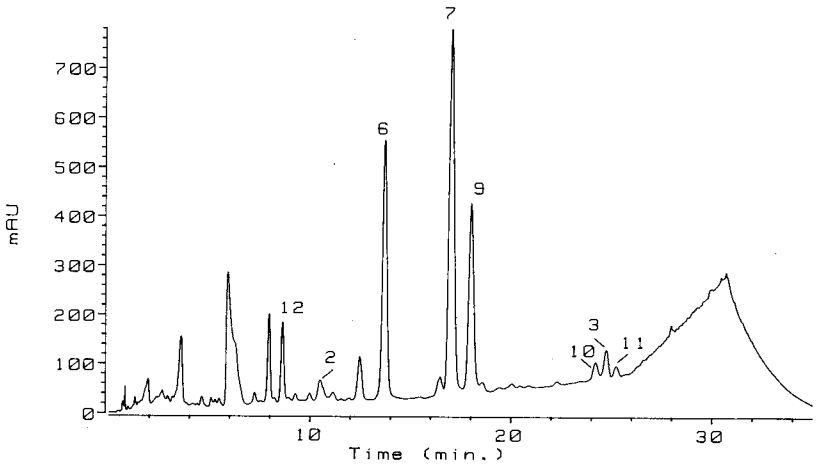
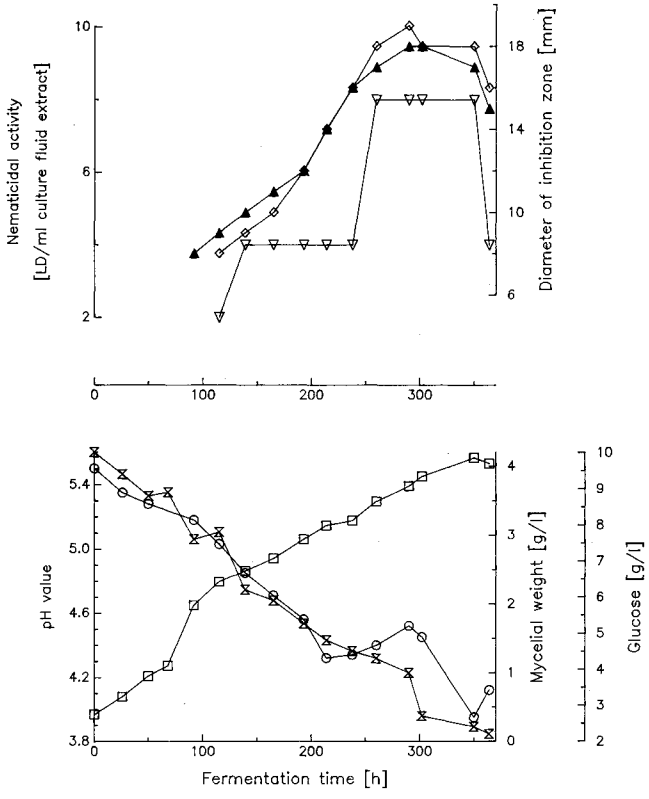


Fig. 2. Fermentation of *Lachnum papyraceum* in 20 liter scale in MGP4 medium with 50 mM $CaBr_2$ added.

○ pH value; □ mycelial dry weight (g/liter); ⧖ glucose; diameter of inhibition zone: ◇ *Bacillus brevis* (mm); ▲ *Nematospora coryli*; ▽ nematicidal activities towards *C. elegans* (amount of LD_{100} contained in 1 ml of culture fluid extract).



with three maxima (first between 216 and 231 nm, second between 265 and 272 nm, and third between 302 and 314 nm) differed from those of the mycorrhizins with two (first between 218 and 248 nm and second between 298 and 305 nm) and the lachnumon derivatives with one maximum (between 251 and 261 nm) but were quite similar to each other, suggesting that the fungus had produced a series of metabolites closely related to each other. The extracts from MGP3 with 5 mM $CaBr_2$ were similar in composition to those from MGP4 with 50 mM $CaBr_2$, but the latter provided higher yields of the metabolites and thus was chosen for fermentations in 20-liter scale. The fermentation diagram is shown in Fig. 2. After 18 days the fermentation was terminated, as biological activities of the culture filtrate extracts started to decrease. During fermentations, both antimicrobial and nematicidal activities of the extracts were much weaker than in case of MGP medium[2]. The culture fluid yielded only rather small amounts of lachnumon (**1**) *e.g.* 14 mg compared to 121 mg from a fermentation in MGP medium, lachnumol A (**2**) 5 mg instead of 50 mg, mycorrhizin A (**3**) 3 mg instead 20 mg, and 2 mg chloromycorrhizin A (**4**) compared to 78 mg. Yields of dechloromycorrhizin A (**5**) were slightly higher *e.g.* 14 mg as compared to 9 mg in MGP medium. Instead, compounds **6**, **7** and **9**～**12** were obtained. The isolation scheme is given in Fig. 3.

The structures of compounds **6**, **7**, **9**, **10**, **11** and **12** are depicted in Fig. 4. The physico-chemical properties and the elucidation of their structures are described in the following paper[4].

Biological Activities

In Tables 1～4, the biological activities of metabolites **6**, **7**, and **9**～**12** are listed. For comparison those of lachnumol A (**2**) and mycorrhizin A are included (**3**). The isocoumarin derivatives were not active in the agar diffusion assay towards filamentous fungi (data not shown) and showed only weak antimicrobial (Table 1), cytotoxic (Table 2) and nematicidal (Table 3) effects in comparison to **2** and **3**. This also explains the low biological activities of the extracts during fermentation in bromide containing media.

Table 4 shows the phytotoxic activities of all metabolites in the plant germination assay. The germination of the monocotyle *Setaria italica* was totally inhibited by 100 μg of the mycorrhizins (**3**～**5**), lachnumon (**1**) and the isocoumarin derivatives **6**, **7**, **9**, and **10**

Fig. 3. Isolation of compounds **1**~**7** and **9**~**12** from the culture fluid of *L. papyraceum* grown in MGP4.

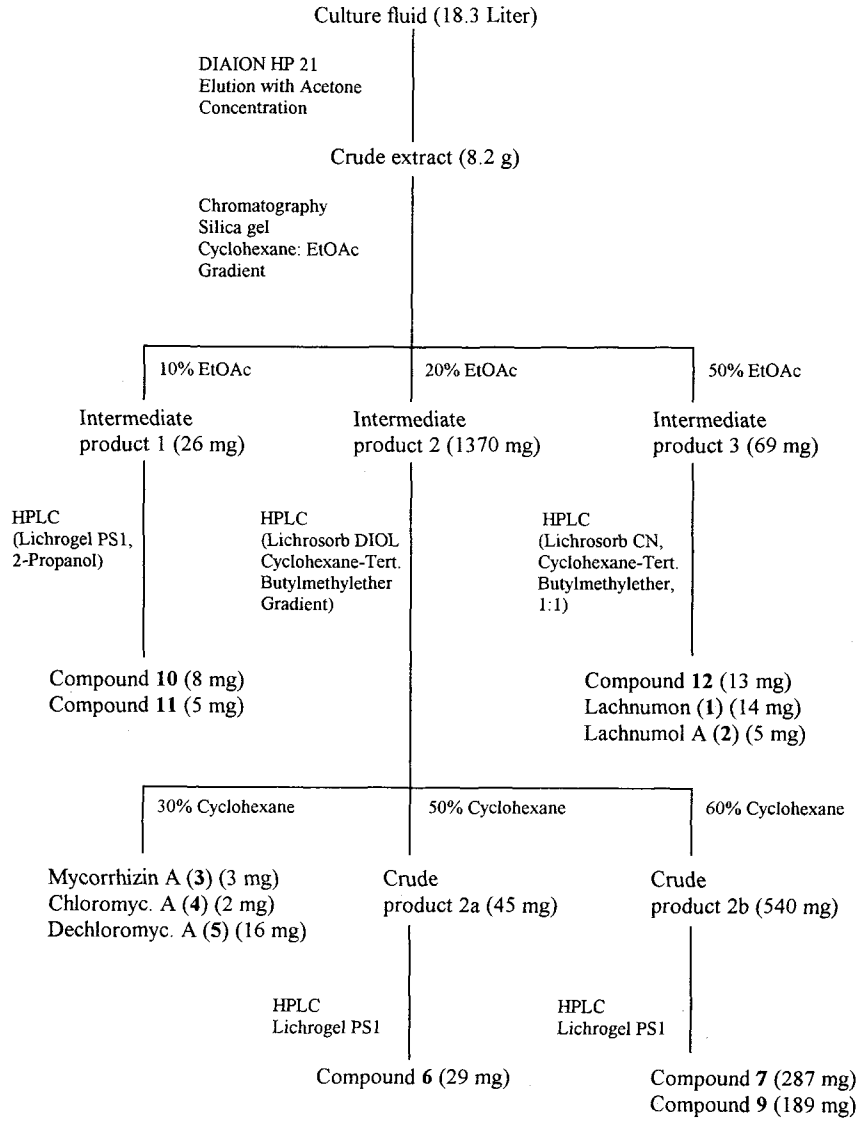
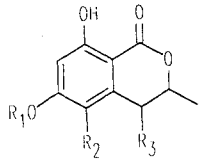


Fig. 4. Structures of compounds **6**, **7** and **9**~**12**.



6 ($R_1=R_2=R_3=H$): 6-hydroxymellein; **7** ($R_1=R_2=H$; $R_3=Cl$): 4-chloro-6-hydroxymellein; **9** ($R_1=R_2=H$; $R_3=Br$): 4-bromo-6-hydroxymellein; **10** ($R_1=CH_3$; $R_2=R_3=H$): 6-methoxymellein; **11** ($R_1=CH_3$; $R_2=H$; $R_3=Cl$): 4-chloro-6-methoxymellein; **12** ($R_1=H$; $R_2=OH$; $R_3=Cl$): 4-chloro-6,7-dihydroxymellein.

whereas the dicotyle *Lepidium sativum* was totally inhibited at 100 μg of the chlorinated mycorrhizins **3** and **4**, lachnumon (**1**), lachnumol A (**2**) and all isocoumarin derivatives (**6**, **7**, **9**~**12**). Unlike in other biological test systems, the isocoumarins **6**, **7**, **9**~**12** showed activities comparable to those of **1**~**5**. Phytotoxic effects of 6-hydroxymellein (**6**)[7] and other related compounds[6,8,9] have already been described.

Up to 150 μg/ml, compounds **6**, **7**, **9**~**12** were not hemolytic towards bovine erythrocytes and had no influence on bovine platelet aggregation[10].

Discussion

Although many other isocoumarin derivatives have been reported from bacteria, fungi, plants, and animals[11], compounds **7**, **9**, **11**, and **12** from *L. papyraceum* have never been isolated from another natural source. 6-Methoxymellein (**10**) has been described as a metabolite of *Aspergillus variecolor*[12], *Sporormia affinis*[13], as a phytoalexin from carrots[14,15], and as a constituent of the bark and roots of *Kigelia pinnata*[16]. It has been reported to be devoid of antibacterial activity[14], but other reports indicated weak antibacterial and antifungal activities[15], these are in agreement with the weak activities in our assays.

Table 1. Antimicrobial activities of compounds **2**, **3**, **6**, **7**, and **9**~**12** in the serial dilution assay towards yeasts and bacteria after 24 hours.

Organism	MIC (μg/ml)							
	2	**3**	**6**	**7**	**9**	**10**	**11**	**12**
Bacteria (Nutrient Broth)								
Acinetobacter calcoaceticus	>100	25	>100	>100	>100	>100	100	>100
Bacillus brevis	25	1	50	100	100	100	100	50
Bacillus subtilis	10	1	50	100	100	100	100	100
Micrococcus luteus	>100	25	100	100	100	>100	100	100
Fungi (YMG medium)								
Candida albicans	10	100	>100	>100	>100	>100	50	100
Nematospora coryli	100	1	100	50	100	100	50	100
Rhodotorula glutinis	25	>100	100	100	100	100	>100	>100
Saccharomyces cerevisiae	10	100	>100	>100	>100	>100	100	100

Table 2. Cytotoxic activities of compounds **2**, **3**, **6**, **7**, and **9**~**12** towards mammalian cell lines.

Cell line	IC_{100} (μg/ml)							
	$\mathbf{2}^{L}$	$\mathbf{3}^{L}$	$\mathbf{6}^{G}$	$\mathbf{7}^{G}$	$\mathbf{9}^{G}$	$\mathbf{10}^{G}$	$\mathbf{11}^{G}$	$\mathbf{12}^{G}$
L 1210	10	0.1	50	>100	100	100	100	100
HL 60	100	0.5	50	100	>100	100	100	50
HeLa S3	10	0.5	100	50	100	100	100	100
BHK 21	100	1.0	>100	100	100	100	>100	100

IC_{100}: L = Compound caused total lysis of the cells.
G = Compound caused only inhibition of growth.

Table 3. Nematicidal activities of compounds **2**, **3**, **6**, **7**, and **9**~**12** towards *Caenorhabditis elegans*.

Compound	ND_{90} (μg/ml)	Compound	ND_{90} (μg/ml)	Compound	ND_{90} (μg/ml)
2	5~10	**7**	100	**11**	100
3	1~2	**9**	100	**12**	50
6	>100	**10**	>100	Ivermectin	0.1

ND_{90} = Concentration causing more than 90% immotility of the nematodes after 18 hours.

Table 4. Phytotoxic activities of compounds **1**~**7** and **9**~**12** in the plant germination assay after 72 hours. Average percentage of germinated seeds (3 × 6 seeds/assay).

Compound	*Setaria italica* % germination		Compound	*Lepidium sativum* % germination	
	10 (μg/paper disc)	100 (μg/paper disc)		10 (μg/paper disc)	100 (μg/paper disc)
1	34	0	**1**	84	0
2	83	67	**2**	84	0
3	50	0	**3**	42	0
4	50	0	**4**	42	0
5	50	0	**5**	67	13
6	13	0	**6**	50	0
7	41	0	**7**	50	0
9	67	0	**9**	33	0
10	50	0	**10**	50	0
11	83	25	**11**	33	0
12	58	33	**12**	41	8

6-Hydroxymellein (**6**) is known from *Azadirachta indica* and several fungi[11] for example *A. terreus*[17], *Gilmaniella humicola*[6,18], and *Pyricularia oryzae*[19]. At high concentrations, compound **6** inhibited the growth of roots of rice seedlings, whereas at low concentrations a stimulation was observed[19]. It was also isolated from infected carrots as a phytoalexin[20] with very weak antifungal activity towards *Cladosporium cucumerium*[21]. Both phytoalexins (**6** and **10**) inhibited germination of plant seeds but had no nematicidal activities. Other phytoalexins, like medicarpin and 4-hydroxymedicarpin were found to be toxic towards nematodes[22].

In the context of the shift in secondary metabolism during fermentations in $CaBr_2$-containing media, the biogenetic scheme for mycorrhizins and related compounds in *Gilmaniella humicola* proposed by CHEXAL *et al.*[6] is very interesting. In *G. humicola* 6-hydroxymellein (**6**) seems to be an intermediate, which is decarboxylated and isoprenylated during mycorrhizin biosynthesis. Some of the proposed intermediates like dechloromycorrhizin A (**5**) have not been isolated from *G. humicola* and from other species producing mycorrhizins except from *L. papyraceum*. Like the mycorrhizins, lachnumols and lachnumon are most probably derived from 6-hydroxymellein. Their biosynthesis branches off prior to the isoprenylation needed for the synthesis of mycorrhizins. This hypothesis is underlined by the fact, that $CaBr_2$ inhibited the synthesis of both lachnumols and mycorrhizins. With these biogenetic pathways almost blocked, 6-hydroxymellein could accumulate and may act as substrate in the halogenation, hydroxylation and methylation steps leading to the compounds described in this paper.

Acknowledgments

We are grateful to the Studienstiftung des deutschen Volkes, Bonn/FRG, for financial support of our work. We thank Dr. KIRSTEN LORENZEN, Univ. Kaiserslautern, for the platelet aggregation assay and R. Reiss for expert technical assistance.

References

1) NEIDLEMAN, S. L. & J. GEIGERT: Biohalogenation. pp. 13~15, Ellis Harwood Ltd. Chichester/UK, 1986
2) STADLER, M.; H. ANKE, W. R. ARENDHOLZ, F. HANSSKE, U. ANDERS, O. STERNER & K. E. BERGQUIST: 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. J. Antibiotics 46: 961~967, 1993
3) STADLER, M.; H. ANKE, O. STERNER & K. E. BERGQUIST: Lachnumon and lachnumol A, new metabolites with nematicidal and antimicrobial activities from the ascomycete *Lachnum papyraceum* (Karst.) Karst. II. Structural elucidation. J. Antibiotics 46: 968~972, 1993
4) STADLER, M.; H. ANKE & O. STERNER: New metabolites with nematicidal and antimicrobila activities from the ascomycete *Lachnum papyraceum* (Karst.) Karst. IV. Structural elucidation of novel isocoumarin derivatives. J. Antibiotics 48: 267~270, 1995
5) ANKE, H.; O. BERGENDORFF & O. STERNER: Assays of the biological activities of guaiane sesquiterpenoids from the fruiting bodies of edible *Lactarius species*. Food Chem. Toxicol. 27: 393~397, 1989
6) CHEXAL, K. K.; CH. TAMM, J. CLARDY & K. HIROTSU: Gilmaniellin and dechlorogilmaniellin, two novel dimeric oxaphenalenones. Helv. Chim. Acta 62: 1785~1803, 1979
7) VENKATASUBBAIAH, P. & W. S. CHILTON: Toxins produced by the dogwood anthracnose fungus *Discula* sp. J. Nat. Products 54: 1293~1297, 1991
8) IWASAKI, S.; H. MURO, K. SAZAKI, S. NOZOE & S. OKUDA: Isolations of phytotoxic substances produced by *Pyricularia oryzae* Cavara. Tetrahedron Lett. 37: 3537~3542, 1973
9) HALLOCK, K.; J. CLARDY, D. S. KENFIELD & G. STROBEL: De-*O*-methylporthin, a phytotoxin from *Drechslera siccans*. Phytochemistry 27: 3123~3125, 1988
10) LAUER, U.; T. ANKE & F. HANSSKE: Antibiotics from Basidiomycetes XXXVII. 2-Methoxy-5-methyl-1,4-benzoquinone, a thromboxane A_2 antagonist from *Lentinus adhaerens*. J. Antibiotics 44: 59~65, 1991
11) BUCKINGHAM, J. (*Ed.*): Dictionary of Natural Products Vol. 1~7, Chapman & Hall, London, 1994
12) DUNN, A. W.; T. J. KING, L. LESSINGER, B. SKLARZ & R. A. JOHNSTONE: Structures of C_{25} compounds from *Aspergillus variecolor*. J. Chem. Soc. Perkin Trans. I: 2113~2117, 1979
13) MCGAHREN, W. J. & L. A. MITSCHER: Dihydrosiocoumarins from a *Sporormia* fungus. J. Org. Chem. 33: 1577~1580, 1968
14) SONDHEIMER, E.: The isolation and identification of 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin from carrots. J. Am. Chem. Soc. 79: 5036~5039, 1957
15) KUROSAKI, F. & A. NISHI: Isolation and antimicrobial activity of the phytoalexin 6-methoxymellein from cultured carrot cells. Phytochemistry 22: 669~672, 1983
16) GOVINDACHARI, T. R.; S. J. PATANKAR & N. VISWANATHAN: Isolation and structure of two new dihydroisocoumarins from *Kigelia pinnata*. Phytochemistry 10: 1603~1606, 1971
17) CURTIS, R. F.; P. C. HARRIES, C. H. HASSALL, J. D. LEVI & D. M. PHILLIPS: The biosynthesis of phenols. Part X. Mutation and radioactive tracer studies relating to the biosynthesis of sulochrin. J. Chem. Soc. C: 1966, 168~174
18) CHEXAL, K. K.; CH. TAMM, J. CLARDY & K. HIROTSU: Gilmicolin and mycorrhizinol, two new metabolites of *Gilmaniella humicola* Barron. Helv. Chim. Acta 62: 1130~1142, 1979
19) IWASAKI, S.; H. MURO, K. SASKI, S. NOZOE & S. OKUDA: Isolations of phytotoxic substances produced by *Pyricularia* oryzae Cavara. Tetrahedron Lett. 1973: 3537~3542
20) COXON, D. T.; R. F. CURTIS, K. R. PRICE & G. LEVETT: Abnormal metabolites produced by *Daucus* carota roots stored under conditions of stress. Phytochemistry 12: 1881~1885, 1973
21) HARDING, V. K. & J. B. HEALE: Isolation and identification of the antifungal compounds accumulating in the induced resistance response of carrot root slices to *Botrytis cinerea*.
22) STADLER, M.; E. DAGNE & H. ANKE: Nematicidal activities of two phytoalexins from *Taverniera abyssinica* A. Rich. (Leguminosae). Planta Medica 60: 550~552, 1994