7-Chloro-4,6-dimethoxy-1(3H)-isobenzofurane and Basidalin: Antibiotic Secondary Metabolites from *Leucoagaricus carneifolia* Gillet (Basidiomycetes)

Thomas Huff and Hans-Georg Kuball

Fachbereich Chemie der Universität, Erwin-Schrödinger-Straße 52, D-67663 Kaiserslautern, Bundesrepublik Deutschland

Timm Anke

Lehrbereich Biotechnologie der Universität, Paul-Ehrlich-Straße 23, D-67663 Kaiserslautern, Bundesrepublik Deutschland

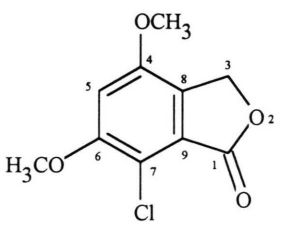
Z. Naturforsch. **49c,** 407–410 (1994); received April 4, 1994

7-Chloro-4,6-dimethoxy-1(3H)-isobenzofurane, Basidalin, *Leucoagaricus carneifolia,* Basidiomycete, Antibiotic

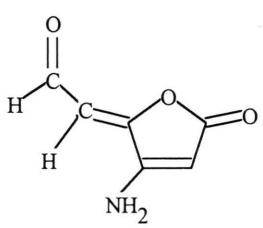
Two antibiotic metabolites were isolated from cultures of *Leucoagaricus carneifolia.* Their structures were elucidated by spectroscopic methods. The first compound, 7-chloro-4,6-dimethoxy-1(3H)-isobenzofurane (**1**) had to our knowledge not been described from natural sources whereas the second, basidalin (**2**), is a known metabolite of *L. naucina* (H. Iinuma *et al.,* 1983). **1** exhibits antibiotic activities with minimal inhibitory concentrations of 20 μg/ml against *Botrytis cinerea,* the most sensitive microorganism.

Introduction

The genus *Leucoagaricus* (Loquin) Sing. is most closely related to the genera *Macrolepiota* and *Leucocoprinus.* It is almost cosmopolitan, most species, however, have been described from America and Africa (R. Singer, 1986). In the course of a screening for new secondary metabolites from basidiomycetes the European species *Leucoagaricus carneifolia* was found to produce two antibiotic and cytotoxic compounds. In the following we wish to describe the fermentation, isolation, structural elucidation and biological characterization of these metabolites which were identified as 7-chloro-4,6-dimethoxy-1(3H)-isobenzofurane (**1**) and basidalin (**2**).



1



2

Materials and Methods

Leucoagaricus carneifolia strain 90352

Mycelial cultures of *L. carneifolia* were obtained from spore prints of fruiting bodies collected in Mölschbach, Germany. Herbarium specimen and mycelial cultures are deposited in the collection of strains, LB Biotechnologie, University of Kaiserslautern.

Reprint requests to Prof. T. Anke.
Telefax: (0631) 2052999.

0939–5075/94/0700–0407 $ 06.00

Fermentation and isolation

For maintenance on agar slants and submerged cultivation *L. carneifolia* was grown in a yeast extract – malt extract – glucose (YMG) medium composed of [g/l]: yeast extract 4, malt extract 10 and glucose 4. A 250 ml culture of *L. carneifolia* grown for 9 days was used to inoculate 20 l of the same medium in a Biolafitte C6 fermentation apparatus. After 16–18 days of fermentation (25 °C, 150 r.p.m., 3.3 l air/min) the mycelia were separated from the culture fluid and discarded. **1** and **2** were adsorbed from the filtrate (15 l) on HP21-resin (Mitsubishi) and eluted with methanol. The solvent was removed under reduced pressure and the oily residue (1.5 g) was applied to a column (silica gel Merck 60; 4×50 cm). **1** and **2** were eluted with cyclohexane–ethyl acetate (8:2; **1**) and (6:4; **2**). Yields: 150 mg of **1** and 400 mg of **2**.

7-Chloro-4,6-dimethoxy-1(3H)-isobenzofurane (**1**)

White powder, m.p. 164.1 °C, soluble in methanol, acetone, retention time 7.2 min [LiChrospher 60 RP-select B (125×4 mm), H_2O–MeCN gradient, 2–100% MeCN in 9 min, flow 1.2 ml/min, detection: UV absorption at 210 nm]; UV (MeOH) λ_{max} nm (ε) 217 (42,000), 257 (11,300), 306 (3600); IR (KBr) cm^{-1} 3430 (w), 3089 (w), 3012 (w), 2956 (w), 2850 (w), 1757 (sst), 1618 (m), 1577 (m), 1478 (s), 1414 (s), 1341 (s), 1120 (sst), 1038 (s), 768 (m); EI-MS, MS, *m/e* 228 (M+, calculated for $C_{10}H_9O_4Cl$ 228.63). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.16 (s, 1H, Ar–H), 5.41 (s, 2H, CH_2), 4.02 (s, 3H, O–CH_3), 3.97 (s, 3H, O–CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100.61 MHz) δ 170.4 (s, C=O), 157.9 and 151.5 (2× s, C–OMe), 128.9 (s, C-8), 125.45 (s, C-9), 121.5 (s, C–Cl), 102.1 (d, CH), 67.9 (t, CH_2), 59.8 and 56.9 (2× q, O–CH_3) ppm; Elemental analysis (calculated for $C_{10}H_9O_4Cl$: C 52.5%, H 4.0%; found: C 52.2%, H 4.2%).

Basidalin (**2**)

Yellowish powder, decomp. 150 °C, soluble in methanol, acetone, retention time 4.8 min [LiChrospher 60 RP-select B (125×4 mm), H_2O–MeCN gradient, 2–100% MeCN in 9 min, flow 1.2 ml/min, detection: UV absorption at 210 nm]; for spectroscopic data see Iinuma *et al.* (1983).

Biological assays

The antimicrobial spectra were determined in the serial dilution assay. [YMG medium, 27 °C for fungi; Nutrient Broth (Difco), 37 °C for bacteria] (Kupka *et al.*, 1979). The cytotoxicity against L 1210 cells (mouse) was determined as described previously (Leonhardt *et al.*, 1987). 10^5/ml L 1210 cells (ATCC CCL219) were incubated in F12 medium containing 1.5% horse serum with or without antibiotic. After 1 and 2 days the cells were counted using a microscope.

Results and Discussion

Structure elucidation

1 has the molecular formula $C_{10}H_9O_4Cl$ on the basis of the mass spectroscopic data *m/e* 228 (M+, calcd 228.63) and NMR spectral analyses. The UV spectrum with maxima at 217, 256 and 306 nm suggested the presence of an aromatic system. The IR spectrum showed the expected absorption of an α,β-unsaturated lactone (1757 cm^{-1}), aryl–alkyl–ether functions (1214 and 1120 cm^{-1} and a chlorine atom bound to an aromatic ring (1038 cm^{-1}). The ^1H NMR spectrum showed four singuletts at 3.97, 4.02, 5.41 and 7.16 ppm with intensities of 3:3:2:1, which were attributed to two methoxy groups, one methylene group and one aromatic hydrogen. These data suggested a chlorodimethoxyphthalide. ^{13}C NMR spectra (Table I) showed the presence of 10 C-atoms. The assignment of the substituents on the aromatic ring was done by application of the increment system given in Kalinowski *et al.* (1984) and by comparison with the data of known compounds. The high field shift of about 17 ppm for C-8 as compared with the unsubstituted phthalide is indicative for a methoxy group at C-4. The dublett at 102.1 ppm ($^1J_{C-H}$ = 168 Hz) shows that C-5 is positioned between the two methoxy groups of the aromatic ring. The signal at 121.5 ppm is in good agreement with the expected value for a chlorinated carbon atom in the vicinity of a methoxy group. The signal at 125.5 could be assigned to C-9. The two signals at 57.0 and 59.9 ppm which split to quartetts in the coupled spectrum appeared in the typical range of methoxy groups. C-4 and C-6, to which the methoxy groups are bound, showed the expected singuletts at 151.5 and 157.8 ppm. The triplett of the

Table I. ^{13}C NMR spectral data of 7-chloro-4,6-dimethoxy-1(3H)-isobenzofurane (**1**).

C-Atom	^{13}C [ppm]	$^{1}J_{H,C}$ [Hz]
1	170.4 (s)	
3	67.9 (t)	145
4	151.5 (s)	
5	102.1 (d)	168
6	157.8 (s)	
7	121.5 (s)	
8	128.9 (s)	
9	125.5 (s)	
10, 11	57.0 (q), 59.9 (q)	145

Table II. Minimal inhibitory concentrations of **1** and **2** in the serial dilusion assay.

Test organism	MIC [μg/ml (μM)] **1**	**2**
Gram-positive bacteria		
Arthrobacter citreus	>100 (44)	>100 (72)
Bacillus brevis	>100 (44)	50 (36)
Bacillus subtilis	>100 (44)	>100 (72)
Corynebacterium insidiosum	>100 (44)	>100 (72)
Mycobacterium phlei	>100 (44)	>100 (72)
Sarcina lutea	>100 (44)	100 (72)
Streptomyces spec. ATCC 23836	>100 (44)	100 (72)
Gram-negative bacteria		
Escherichia coli	>100 (44)	>100 (72)
Pseudomonas fluorescens	>100 (44)	>100 (72)
Yeasts and Fungi		
Nadsonia fulvescens	>100 (44)	>100 (72)
Rhodotorula glutinis	>100 (44)	>100 (72)
Saccharomyces cerevisiae S288	>100 (44)	>100 (72)
Saccharomyces cerevisiae is1	>100 (44)	>100 (72)
Absida glauca	>100 (44)	>100 (72)
Botrytis cinerea	20 (8.8)	100 (72)
Eurotium cristatum	>100 (44)	>100 (72)
Paecilomyces varioti	>100 (44)	>100 (72)
Penicillium notatum	>100 (44)	>100 (72)
Phytophthora infestans	>100 (44)	>100 (72)
Ustilago nuda	>100 (44)	>100 (72)
Verticillium	>100 (44)	>100 (72)
Zygorhynchus moelleri	>100 (44)	>100 (72)

CH_2 group (C-3) appeared at 67.9 ppm ($^{1}J_{C-H}$ = 145 Hz). The signal at 170.4 ppm was assigned to C-1.

The structure **1** deduced from our spectroscopic data is isomeric to 4-chloro-5,7-dimethoxy-1(3H)-isobenzofurane, a compound synthesized previously by Mirrington *et al.* (1966). A direct comparison of their ^{1}H NMR data yielded additional proof for structure **1.** Phthalides are among the more common natural compounds and a variety of biological activities have been reported for these metabolites [W. B. Turner and D. C. Aldrich, 1983, Dictionary of Natural Products (J. Buckingham, ed.), 1992]. Mycophenolic acid, one of the first known secondary metabolites isolated from fungi, is a phthalide derivative (for review see V. Betina, 1989).

Basidalin [4-amino-5-(formylmethylene)-2(5H)-furanone] was identified (T. Huff, 1993) by direct comparison with the spectral data published by Iinuma *et al.* (1983).

Biological activities

The antimicrobial activities of **1** and **2** in the serial dilution assay are shown in Table II. **1** exhibits its selective activity against *Botrytis cinerea* with a minimal inhibitory concentration (MIC) of 20 μg/ml. All other fungi and bacteria were not inhibited by 100 μg/ml. No cytotoxic activities against L 1210 cells (mouse, lymphocytic leukemia) were detected. **2** shows weak antibiotic activities towards *B. brevis, M. luteus, Streptomyces* sp. ATCC 23836, and *B. cinerea.* Iinuma *et al.* (1983) had reported weak antibacterial activity towards *Aeromonas salminicola* and *Vibrio anguillarum.* The observed cytotoxic activities of basidalin (1 μm/ml, L 1210 cells) are in good agreement with the data of Iinuma *et al.* (1983).

Acknowledgements

We thank Dr. F. Hansske and Dr. H. Hindermayr, Boehringer, Mannheim, Germany, for mass spectroscopic analysis.

Betina V. (1989), Mycotoxins. Elsevier, Amsterdam.
Dictionary of Natural Products (J. Buckingham, ed.). Chapman and Hall, London 1992.
Huff T. (1993), Dissertation, Universität Kaiserslautern.
Iinuma H., Nakamura H., Naganawa H., Masuda T., Takano S., Takeuchi T., Umezawa H., Iitaka Y. and Obayashi A. (1983), Basidalin, a new antibiotic from basidiomycetes. J. Antibiotics **36,** 448–450.
Kalinowski H. O., Berger S. and Braun S. (1984), ^{13}C-NMR-Spektroskopie. Georg Thieme Verlag, Stuttgart, New York.
Kupka J., Anke T., Oberwinkler F., Schramm G. and Steglich W. (1979), Antibiotics from Basidiomycetes VII. Crinipellin, a new antibiotic from the basidiomycetous fungus *Crinipellis stipitaria* (Fr.) Pat. J. Antibiotics **32,** 130–135.
Leonhardt K., Anke T., Hillen-Maske E. and Steglich W. (1987), 6-Methylpurine, 6-methyl-9-β-D-ribofuranosyl purine, and 6-hydroxymethyl-9-β-D-ribofuranosyl purine as antiviral metabolites of *Collybia maculata* Basidiomycetes). Z. Naturforsch. **42c,** 420–424.
Singer R. (1986), The Agraricales in Modern Taxonomy. Koeltz Scientific Books, Königstein.
Turner W. B. and Aldrich D. C. (1983), Fungal Metabolites II. Academic Press, London.