Mollisianitrile, a New Antibiotic from *Mollisia* sp. A59–96

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Mollisianitrile (**1**), a new antibiotic was isolated from the fermentation broth of *Mollisa* sp. A59–96 together with the two known isocoumarins **2** and **3**. **1** exhibited antimicrobial, cytotoxic, and phytotoxic activities. **1** contains a reactive propiolonitrile moiety which is believed to be responsible for its antibiotic activities. Upon incubation with L-cysteine the biological activity was lost.

Key words: Mollisia, Mollisianitrile, Antimicrobial and Cytotoxic Activities

Introduction

The genus *Mollisia* is widely spread and its species have been described as endophytes (Kowalski and Kehr, 1996) and saprophytes. During a screening of fungal extracts for new metabolites with antimicrobial and cytotoxic activities, extracts of *Mollisia* sp. A 59–96 inhibited the growth of bacteria, fungi, and mammalian cells. So far, few compounds have been described from the genus *Mollisia*, among them the chlorinated metabolites KS-504a–d, the zaragozic acids, benesudon, and mollisin (Nakanishi *et al*., 1989; Thines *et al*., 1997; van der Kerk and Overeem, 1957). This report describes the production, structure elucidation, and the biological activities of the active principle mollisianitrile (**1**).

Materials and Methods

Producing organism

The mycelial culture of *Mollisia* sp. A59–96 was obtained from spores. The fruiting bodies, growing on a dead twig, were collected in France. The genus was identified by macroscopic and microscopic characteristics. Voucher specimen and mycelial cultures are deposited in the culture collection of the IBWF e.V., Kaiserslautern, Germany. For maintenance on agar slants the fungi were grown on YMG medium (g/l): yeast extract (4), malt extract (10), glucose (10), and 2% agar for solid media. The pH was adjusted to 5.5.

*Fermentation of Mollisia sp. A59–96 and isolation of compounds **1**, **2**, and **3***

The fungus was grown in YMG medium. Fermentations were carried out in a Biolafitte C6 fermentor containing 20 l of medium with aeration (3 l air/min) and stirring (120 rpm) at room temperature. A well-grown culture (in 250 ml YMG medium) in a 500 ml Erlenmeyer flask (grown at 22 °C and 120 rpm) was used as inoculum. When the glucose was used up (approx. after 12 d), the culture fluid was separated from the mycelium by filtration. The culture broth was extracted with an equal volume of EtOAc, the organic phase dried over Na_2SO_4 and concentrated *in vacuo*.

The crude extract (1 g) was applied onto a column (2.5 × 25 cm) containing silica gel (Merck 60, 0.063–0.2 mm) and eluted with cyclohexane, cyclohexane/ethyl acetate and ethyl acetate. An enriched product (67 mg) was obtained after elution with cyclohexane/ethyl acetate (3:1). Preparative HPLC yielded 7.9 mg of **2** eluting at 25% MeCN. For preparative HPLC a Jasco Model PU-1586 instrument with a multiwavelength detector MD-910 was used (column: Merck RP 18, 7 μm; 250 × 25 mm; gradients: H_2O/MeCN; flow: 15 ml/min). Chromatography on silica gel in cyclohexane/ethyl acetate (1:1) yielded 169 mg of a second intermediate product. From this 12.3 mg of mollisianitrile (**1**) were obtained by preparative HPLC (see above) eluting at 48% MeCN. The enriched prod-

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uct (181 mg) obtained after elution with ethyl acetate yielded 7 mg of **3** after preparative HPLC (see above) eluting at 40% MeCN.

Spectroscopic characterization and structure elucidation

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probe head equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, containing 5% CD_3OD and the solvent signals of $CHCl_3$/$CDCl_3$ (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm and the coupling constants (J) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{CH}$ = 145 Hz and $^nJ_{CH}$ = 10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). Mass spectra were recorded with a LC-MS (HP 1100; APCI, positive/negative mode) and a Micromass Q-TOF MICRO instrument (HR electrospray spectra), while the UV and IR spectra were recorded with a Perkin-Elmer λ 16 and a Bruker IFS 48 spectrometer.

Mollisianitrile [(3-(3,5-dihydroxy-4-methoxyphenyl)propiolonitrile, **1**] was obtained as a yellowish oil without optical activity. – UV (MeOH): λ_{max} (log ε) = 222 (4.33), 288 nm (4.20). – IR (KBr): ν = 3355, 2943 2271, 2147, 1591, 1529, 1437, 1379, 1319, 1211, 1054, 994, 840, 621 cm^{-1}. – ^{1}H NMR (500 MHz): δ = 6.68 (2H, s, 2-H), 3.92 (3H, s, 4-Ome). – ^{13}C NMR (125 MHz): δ = 149.8 (C-3), 138.8 (C-4), 113.1 (C-2), 112.2 (C-1), 105.6 (C-3′), 83.5 (C-1′), 61.7 (C-2′), 60.8 (4-Ome). – HRES-IMS: m/z = 190.0512 [M+H$^+$] ($C_{10}H_8NO_3$ requires 190.0504).

Biological assays

Antimicrobial activities were determined using the serial dilution assay (Anke *et al.* 1989). Inhibition of growth of germinated seeds of *Setaria italica* and *Lepidium sativum* was tested according to Anke *et al.* (1989). Nematicidal activity was measured as described by Stadler *et al.* (1994).

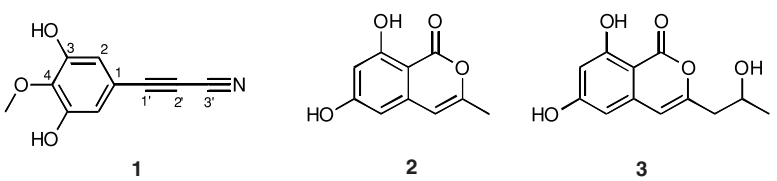
Cytotoxic activity was assayed as described previously (Zapf *et al.*, 1995) with slight modifications. Jurkat (ATCC TIB 152) and COLO-320 cells (DSMZ ACC144) were grown in RPMI 1640 medium (GIBCO, BRL), MDA-MB-231 (ATCC HTB26) and MCF-7 (ATCC HTB 22) cells in D-MEM medium (GIBCO, BRL), supplemented with 10% fetal calf serum (FCS) (GIBCO, BRL), 65 μg/ml of penicillin G and 100 μg/ml of streptomycin sulfate. The assays contained 1×10^5 cells/ml medium. In addition to a microscopic examination after 24, 48, and 72 h, the effect on the growth of monolayer cell lines was measured by Giemsa staining. The viability of suspension cells was measured in a test based on XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide].

Results and Discussion

Isolation and structure determination

The three compounds **1**, **2** and **3** were isolated from the culture fluid of *Mollisia* sp. A59–96 as described above and their structures determined by spectroscopic techniques.

The structure of the new compound mollisianitrile (**1**) (Fig. 1) was elucidated based on spectroscopic data. High resolution MS experiments revealed that the elemental composition of **1** is $C_{10}H_7NO_3$, giving it an unsaturation index of 8, and only two signals (integrating for five protons) were visible in the ^{1}H NMR spectrum. As the ^{13}C NMR spectrum only contained 8 signals, a symmetry is present in the molecule. The unsaturated protons gave HMBC correlations to their own car-



1 2 3

Fig. 1. Structures of mollisianitrile (**1**), 6,8-dihydroxy-3-methylisocoumarin (**2**), and 6,8-dihydroxy-3-(2-hydroxypropyl)isocoumarin (**3**).

bon atom (C-2), C-3, C-4 and C-1′, while the methoxy protons gave HMBC correlations to C-4. Together with the typical chemical shifts of the carbon atoms and protons involved, this suggests the presence of a 3,5-dihydroxy-4-methoxybenzene, with C-1′ attached to C-1. This would account for $C_7H_7O_3$ and 4 unsaturations, leaving C_3N and 4 unsaturations for the C-1 substituent. The two distinct IR absorption bands at 2271 and 2147 cm^{-1} suggest the presence of both an unsymmetrical alkyne as well as a nitrile functionality, and the ^{13}C NMR data are in agreement with this.

The two isocoumarins 6,8-dihydroxy-3-methylisocoumarin (**2**) and 6,8-dihydroxy-3-(2-hydroxypropyl)isocoumarin (**3**) are known compounds. **2** was isolated from the marine fungus *Keissleriella* sp. YS4108, the basidiomycete *Crinipellis stipitaria*, *Ceratocystis minor* and other fungi (Liu *et al*., 2002; Lange *et al*., 1995; Hedge *et al*., 1989). **3** was isolated among others from *Daldinia concentrica*, *Aspergillus ochraceus*, *Rhynchosporium orthosporum* and *Drechslera siccans* (Quang *et al*., 2002; Harris and Mantle, 2001; Ishihara *et al*., 1989; Hallock *et al*., 1988). **2** and **3** (Fig. 1) were identified by comparing their NMR data with those reported.

Biological properties

The antimicrobial spectrum of **1** is shown in Table I. In the serial dilution assay mollisianitrile exhibited antibacterial and antifungal activities. **1** showed cytotoxic activity against all cell lines tested (Table II). The IC_{50} values for monolayer cells were 5 μg/ml and for suspension cells 0.5 to 1 μg/ml. For the two known compounds **2** and **3** only weak cytotoxic activities were measured. Mollisianitrile was phytotoxic against *Lepidium sativum* and *Setaria italica*. 70% inhibition of growth was observed for seedlings of *Lepidium sativum* and 60% inhibition for seedlings of *Setaria italica* at 100 μg/ml. No nematicidal activity of **1** against *Caenorhabditis elegans* or *Meloidogyne incognita* was detected at concentrations up to 100 μg/ml.

After 5 min incubation of mollisianitrile with L-cysteine, a ninhydrin positive adduct was detected by TLC and the antibacterial activity against *Bacillus subtilis* was lost. It can be assumed that the reactive triple bond is responsible for most of the observed biological activities.

Table I. Antimicrobial activity of **1** in the serial dilution assay.

Testorganism	MIC [μg/ml]
Bacteria	
Arthrobacter citreus	20
Bacillus brevis	10
Bacillus subtilis	10
Corynebacterium insidiosum	10
Mycobacterium phlei	10s
Enterobacter dissolvens	100s
Escherichia coli K12	20
Pseudomonas fluorescens	5
Fungi	
Nematospora coryli	5
Absidia glauca +	>100
Absidia glauca –	>100
Ascocyta pisi	>100
Aspergillus ochraceus	>100
Fusarium fujikuroi	>100
Fusarium oxysporum	>100
Mucor miehei	20
Paecilomyces variotii	25
Penicillium islandicum	100
Penicillium notatum	100
Zygorhynchus moelleri	>100

s, Bacteriostatic.

Table II. Cytotoxic activities of compounds **1**–**3**.

Cell line	IC_{50} [μg/ml]		
	1	**2**	**3**
COLO-320	0.5	>50	n.t.
HL-60	1	>50	n.t.
L1210	0.5	50	n.t.
Jurkat	0.5	50	50
MDA-MB-231	5	>50	n.t.
MCF-7	5	>50	n.t.

n.t., Not tested.

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