Xylaramide, a New Antifungal Compound, and Other Secondary Metabolites from *Xylaria longipes*

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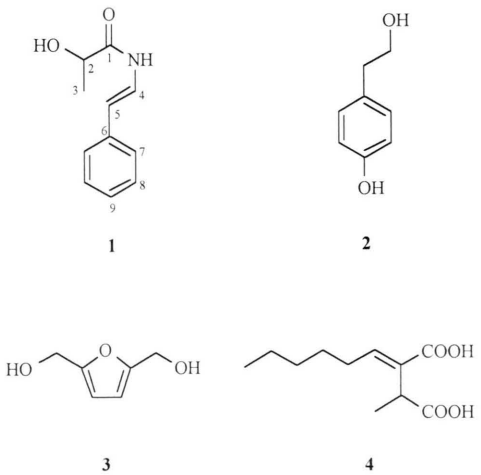
Xylaramide (**1**), possessing potent antifungal activity towards *Nematospora coryli* and *Saccharomyces cerevisiae*, was isolated from the culture fluids of the wood-inhabiting ascomycete *Xylaria longipes* together with tyrosol (**2**), 2,5-bis(hydroxymethyl)furan (**3**) and 2-hexylidene-3-methylsuccinic acid (**4**). The latter has been known as a *Xylaria* metabolite for many years. Compounds **2** and **3** have been previously reported from other fungi, whereas **1** is a new natural N-(2-phenylethenyl)-2-hydroxypropanamide. The isolation, structure determination and biological properties of xylaramide are described. The biological activities of the other compounds are included.

Introduction

The genus *Xylaria* belongs to the sphaeriaceous genera of ascomycetes, which are world-wide distributed. Most of them are wood-inhabiting fungi, some causing white-rot (Catechside and Mallett, 1991). The recent interest in *Xylaria* species has focused on their phytopathogenicity (Nilsson *et al.*, 1989) and their production of enzymes (Wei *et al.*, 1992). The latter reflects their natural habitat and ecological role and suggests a possible application in the biotechnological degradation of lignin and other biotransformations (Siebers-Wolff *et al.*, 1993). Investigations of their secondary metabolism have yielded quite different compounds, e.g. succinic acid derivatives (Anderson *et al.*, 1985), cytochalasins (Edwards *et al.*, 1991, Dagne *et al.*, 1994), terpenoids (Schneider *et al.*, 1995) and polyketides (O'Hagan *et al.*, 1992). The production of certain secondary metabolites was used to determine the inter-generic relationship within the family of the Xylariaceae (Whalley *et al.*, 1986, Whalley and Edwards, 1995).

During a screening of higher fungi for the production of bioactive compounds, extracts of the culture fluids of *Xylaria longipes*, A19–91, showed antifungal activity. First investigations revealed, that none of the compounds already known from fungi of the genus *Xylaria* was responsible for the antifungal activity. The active constituents were isolated from the culture broths of 20-l fermentations by bioassay-guided fractionation. Besides xylarin, a new antifungal diterpene (Schneider *et al.*, 1995), three compounds with antifungal activity were obtained. A fourth compound crystallized during the isolation of the other compounds. The production, isolation, structural elucidation and biological characterization of the four metabolites



1 **2** **3** **4**

Fig. 1. Structures of *Xylaria longipes* metabolites.

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shown in Figure 1, of which xylaramide (**1**) is a new compound, are reported.

Experimental

Producing Organism

Fruiting bodies of *Xylaria longipes*, A19–91, were collected in Lescun, France in 1991. A herbarium specimen and mycelial cultures are deposited at the LB Biotechnology, University of Kaiserslautern.

Fermentation

For maintenance on agar slants (with 2% agar) and submerged cultivation, the strain was grown on YMG medium composed of (g/l): glucose 4, malt extract 10, yeast extract 4. The pH was adjusted to 5.5 before autoclaving. Fermentations were performed in a Biolafitte C-6 apparatus containing 20 l of YMG medium with agitation (120 rpm) and aeration (3.2 l/min) at 22 °C. 200 ml of a well grown culture in the same medium were used as inoculum. The antifungal activity during fermentation was measured in the agar plate diffusion assay with *Nematospora coryli* as test organism.

Isolation

After ten days of fermentation of *Xylaria longipes*, A19–91, the culture fluid (18 l) was separated from the mycelia by filtration and passed through a column with Mitsubishi HP21 resin (column size: 6.5 x 30 cm). The resin was washed with water, and the adsorbed materials were eluted with 1.2 l of acetone. Evaporation of the solvent yielded a crude extract (2.6 g), which was fractionated on a silica gel column (Merck 60; 60–200 μm diameter, 110 g) with cyclohexane – EtOAc (3:7) as eluant. Final purification of the antifungal compounds was achieved by preparative HPLC on Merck Lichrosorb Diol (250 x 25 mm, 7 μm). Elution with cyclohexane – *tert*-butylmethyl ether 4:6 yielded compounds **1**, **2** and **4**, while compound **3** eluted with cyclohexane – *tert*-butylmethyl ether 3:7. From 18 l of culture 1.4 mg of xylaramide (**1**), 14.9 mg of compound **2**, 2.9 mg of compound **3**, and 25 mg of compound **4** were obtained.

Spectroscopy

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature in $CDCl_3$ with a Bruker ARX 500 spectrometer with an inverse 5 mm probe equipped with a shielded gradient coil. COSY, HMQC and HMBC experiments were performed with gradient enhancements using sine shaped gradient pulses, and for the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{CH}$=145 Hz and $^2J_{CH}$= 10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). The chemical shifts are given in ppm (with the solvent peaks at 7.26 and 77.0 ppm serving as reference) and the coupling constants *J* in Hz. EI mass spectra were recorded by a JEOL SX102 spectrometer at 70 eV. The IR spectrum was recorded with a Perkin-Elmer 298 spectrometer, the UV spectrum with a Varian Cary 219 spectrometer, and the optical rotations were measured with a Perkin-Elmer 141 polarimeter at 22°C.

Xylaramide (**1**), N-(2-phenylethenyl)-2-hydroxypropanamide, was obtained as a colorless oil. $[\alpha]_D$ 0 ° (c 0.2 in chloroform). UV (methanol) λ_{max} (ε): 279 nm (10,600). IR (KBr): 3400, 2925, 1640, 1520, 1280, 1120, 950, 760, and 690 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz), δ, mult. *J* (Hz): 8.39, brd, $J_{4\text{-}NH}$=11, N-H; 7.47, dd, J_{4-5}=14.6, $J_{4\text{-}NH}$=11, 4-H; 7.32, d, J_{7-8}=7.2, 7-H$_2$; 7.28, dd, J_{7-8}=7, J_{8-9}=7, 8-H$_2$; 7.18, t, J_{8-9}=7, 9-H; 6.19, d, J_{4-5}=14.6, 5-H; 4.37, q, J_{2-3}=6.9, 2-H; 1.51, d, J_{2-3}=6.9, 3-H$_3$. ^{13}C NMR ($CDCl_3$, 125 MHz), δ: 171.5 C-1; 135.9 C-6; 128.7 C-8; 126.8 C-9; 125.6 C-7; 121.8 C-4; 114.0 C-5; 68.6 C-2; 21.2 C-3. MS (EI, 70 eV), *m/z*: 191.0955 (M$^+$, 56%, $C_{11}H_{13}NO_2$ requires 191.0946), 119.0748 (100%, C_8H_9N requires 119.0735), 118 (36%), 91 (22%), 84 (21%), 45 (23%).

2,5-Bis(hydroxymethyl)furan (**3**) was obtained as a colorless oil. UV (methanol) λ_{max} (ε): 224 nm (6,100). IR (KBr): 3400, 2930, 1050, and 800 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz), δ, mult.: 6.16, s, 3-H and 4-H; 4.49, -C**H**$_2$OH; 2.62, brs, -OH. ^{13}C NMR ($CDCl_3$, 125 MHz), δ: 154.1 C-2 and C-5; 108.2 C-3 and C-4; 57.0 -**C**H$_2$OH. MS (EI, 70 eV), *m/z*: 128.0461 (M$^+$, 82%, $C_6H_8O_3$ requires 128.0473), 111 (31%), 97 (100%), 84 (40%), 69 (40%).

According to their spectroscopic data, compounds **2** and **4** were identified as tyrosol and 2-hexylidene-3-methylsuccinic acid (Devys *et al.*, 1976, Anderson *et al.*, 1985).

Biological assays

The assays for antimicrobial activity were performed as described previously (Anke *et al.*, 1989) using an inoculum of 1x 10^5 cells or spores/ml. Cytotoxic activity against L1210 cells (ATCC CCl 219, mouse), HL60 cells (ATCC CCL240, human),

BHK 21 cells (ATCC CCL 10, hamster) and HeLa S3 cells (ATCC CCL2.2, human) was measured as described previously (Zapf *et al.*, 1995). The phytotoxic effect on germination of *Setaria italica* and *Lepidium sativum* were evaluated according to Anke *et al.*, (1989). The nematicidal activity against *Caenorhabditis elegans* and *Meloidogyne incognita* were carried out as described by Stadler *et al.* (1993) and Anke *et al.* (1995). The lytic activity towards bovine erythrocytes was measured as described previously (Kuschel *et al.*, 1994).

Results and Discussion

From fermentations of *Xylaria longipes*, A19–91, four natural products were obtained. The most potent antifungal metabolite (**1**) is a new compound for which we propose the name xylaramide. Its structure was determined by NMR spectroscopy and mass spectrometry. The EI mass spectrum contains essentially two ions, the molecular ion at *m/e* 191 and the base peak at *m/e* 119. High resolution EIMS measurements suggest that the elemental composition of xylaramide (**1**) is $C_{11}H_{13}NO_2$ and that the composition of the base peak is C_8H_9N. The ^1H and ^{13}C NMR spectra showed typical signals for a monosubstituted benzene, a trans double bond, a carbonyl group, and a 1-hydroxyethyl group. A broad doublet at 8.4 ppm for an exchangeable proton was observed in the ^1H NMR spectrum, the signal disappeared slowly when D_2O was added to the NMR tube and did not show any correlation in the HMQC spectrum. The structure of xylaramide (**1**) was determined by long range ^1H-^{13}C correlations: 9-H correlated to C-7, 8-H to C-6 and C-8, 7-H to C-5 and C-9, and 5-H correlates to C-4 and C-7, suggesting that C-5 is attached directly to C-6. H-4, which couples to both H-5 and to the exchangeable proton with the coupling constants 14.6 and 11 Hz, respectively, gives HMBC correlations to C-1, C-5 and C-6. 2-H as well as 3-H_3 correlate to C-1, and the suggested structure is the only conceivable that fits the spectroscopic data.

Tyrosol (**2**) has been isolated from higher fungi and plants like *Ceratocystis* species (Ayer *et al.*, 1986), *Gibberella fujikuroi* (Cross *et al.*, 1963), *Pyricularia oryzae* (Devys *et al.*, 1976), *Candida albicans* (Lingappa *et al.* 1969) and *Ligustrum ovalifolium* (Veer *et al.*, 1957).

The furan **3** was recently reported as a metabolite of *Phellinus linteus*, a wood-inhabiting basidiomycete (Song *et al.*, 1994). The paper is in Korean, and the spectroscopic data of the furan **3** are therefore given in the Experimental section.

Compound **4** is common among secondary metabolites produced by fungi of the Xylariaceae, but different optical rotations have been published. Whereas for the product of *Xylaria longipes* a negative rotation (– 89 °, c 1.0 methanol) has been reported (Anderson *et al.*, 1985), the rotation of the sample isolated from *Xylaria longipes*, A19–91, in this investigation was + 71 ° (c 1.3 chloroform). Thus our strain produced the enantiomer.

The biological activities of the isolated *Xylaria* metabolites towards fungi, bacteria, cells, plant germination, nematodes and bovine erythrocytes, were investigated. The antifungal activity (Table I) of xylaramide (**1**) is high towards *Nematospora coryli* and *Saccharomyces cerevisiae* is 1, a permeation deficient mutant. The other yeasts and the following filamentous fungi were not sensitive at concentrations up to 100 µg/ml: *Fusarium oxysporum*, *Mucor miehei*, *Paecilomyces variotii*, *Penicillium notatum* and *Ustilago nuda*. The difference in sensitivity of the two *Saccharomyces cerevisiae* strains indicates that xylaramide can not enter the cells of most yeasts. Whether this holds for filamentous fungi is not known. A degradation and inactivation of xylaramide by a dipeptidase is also conceivable. The selectivity and the lack of apparent reactive chemical functionalities in the structure of xylaramide (**1**) obviously makes it interesting for further studies. The other metabolites exhibited only weak antifungal activities against yeasts and did not affect the growth of the filamentous fungi mentioned above. There was no or only a weak cytotoxic activity of the compounds

Table I. The antifungal activity (MIC) of compound **1**–**4** in the serial dilution assay. (Size of inoculum: $1x10^5$ cells or spores/ml).

Organism	Compound:	MIC [µg/ml] **1**	**2**	**3**	**4**
Yeasts:					
Nadsonia fulvescens		>100	>100	>100	>100
Nematospora coryli		1	100	25s,50	>100
Rhodotorula glutinis		>100	>100	>100	>100
Saccharomyces cerevisiae S 288 c		>100	>100	>100	>100
S. cerevisiae is 1		5	100s	100s	>100

s: Fungistatic, the growth restarted after removal of the compound.

against BHK 21 cells, HeLa S3 cells, HL60 cells and L1210 cells. Whereas xylaramide (**1**) was cytotoxic against BHK 21 cells, HeLa S3 cells and HL60 cells at 100 μg/ml, the furan **3** only affected HL60 cells. For all compounds no antibacterial activity (up to concentrations of 100 μg/ml) was detected with the following organisms: *Acinetobacter calcoaceticus*, *Arthrobacter citreus*, *Bacillus brevis*, *B. subtilis*, *Corynebacterium insidiosum*, *Escherichia coli* K12, *Micrococcus luteus*, *Mycobacterium phlei*, *Salmonella typhimurium* TA 98, and *Streptomyces* spec. ATCC 23836. The plant germination of *Lepidium sativum* and *Setaria italica* was not affected by all compounds at concentrations up to 600 μg/ml but the growth of *Lepidium sativum* seedlings was reduced by the compounds **1**, **2** and **4**. The phytotoxic effect of compound **2** has been previously reported (Devys *et al.*, 1976). At 100 μg/ml none of the compounds showed nematicidal activity towards *Caenorhabditis elegans* and *Meloidogyne incognita* and they had no lytic effect on bovine erythrocytes.

As shown in this publication *Xylaria longipes*, A19–91, is a rich source of secondary metabolites which are derived from different biochemical pathways. Besides the compounds described in this publication, xylarin, a diterpene connected to an unusual glucuronic acid moiety (Schneider *et al.*, 1995) and mellein (Schneider, unpublished) were isolated. The production of secondary metabolites with high antifungal activities by *Xylaria longipes* could be a useful tool against competitors in its ecological niche.

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