Cytotoxic *α*-Pyrones from *Xylaria hypoxylon*

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Two new *α*-pyrone derivatives, xylarone (**1**) and 8,9-dehydroxylarone (**2**) possessing cytotoxic activities, were isolated from the culture fluid of submerged cultures of the ascomycete *Xylaria hypoxylon*, strain A27-94. Their structures were elucidated by spectroscopic methods.

Key words: Xylarone, 8,9-Dehydroxylarone, *α*-Pyrone, *Xylaria hypoxylon*

Introduction

Fungi of the genus *Xylaria* are known to be a rich source of secondary metabolites, among them are succinic acid derivatives (Anderson and Edwards, 1985), cytochalasins (Dagne *et al.*, 1994) and the more common melleins (Whalley and Edwards, 1995). Many of the metabolites possess biological activity, and remarkable antifungal activities have been reported for xylarin (Schneider *et al.*, 1995) and xylaramide (Schneider *et al.*, 1996). *Xylaria* species occur worldwide from arctic to tropic regions where they are especially abundant and occupy ecological diverse habitats. They are often isolated as endophytes and some species are phytopathogenic, for example *Xylaria arbuscula* (Whalley, 1996). During a screening of fungi for the production of biologically active metabolites, extracts of the culture fluid of *Xylaria hypoxylon*, strain A27-94, were found to possess cytotoxic activities. A preliminary investigation indicated that compounds other than cytochalasins were responsible for this activity. The cytotoxic principles were therefore isolated by bioassay-guided fractionation and two cytotoxic compounds, **1** and **2**, were obtained. Their structures were determined by spectroscopic methods. This paper reports the fermentation of strain A27-94, the isolation and structure determination of **1** and **2**, as well as the biological activities of the new metabolites.

Materials and Methods

General experimental procedures

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probe equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (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 (HRESI) were recorded with a Micromass Q-TOF MICRO instrument. FT-IR spectra were recorded with a Bruker IFS-48 spectrometer and UV spectra with a Perkin-Elmer Lambda 16 spectrometer.

Producing organism

Fruiting bodies of *Xylaria* species, A27-94, growing on wood in Canada (vicinity of Vancouver) were collected in 1994. Mycelial cultures were obtained from interior tissues of surface-sterilized fruiting bodies. Herbarium specimen and mycelial cultures are deposited in the culture collection of the institute of biotechnology and drug research (IBWF), Kaiserslautern, Germany. Morphology of the fruiting bodies resembled *Xylaria hypoxylon*, but unfortunately no perithecia were found (Breitenbach and Kränzlin, 1984). However, its ITS sequence showed a homology of 99.6% with *Xylaria hypoxylon* (DQ491487).

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Fermentation of Xylaria hypoxylon and isolation of the compounds

For submerged cultivation strain A27-94 was grown at 22–24 °C in YMG medium (yeast extract 4 g/l, malt extract 10 g/l, glucose 10 g/l, the pH value was adjusted to 5.5 before autoclaving) in a 20 l-fermenter (Biolafitte). A well grown shake culture (250 ml) in the same medium in a 500 ml-Erlenmeyer flask was used as inoculum. The fermentation was carried out with agitation (130 rpm) and aeration (3 l/min) for 8 d. Mycelia containing no active metabolites were discarded after filtration. The compounds were isolated from the culture broth (16 l) by adsorption onto HP 21 resin (Mitsubishi) and elution with MeOH (1.5 l). The crude extract (1.1 g) obtained by concentration was applied onto silica gel (Merck 60, 0.063 ~ 0.2 mm, 60 g). Elution with cyclohexane/EtOAc (6:1) yielded 61.4 mg. Final purification was achieved by preparative HPLC (Merck, Lichrosorb RP 18, 7 μm, 250 × 25 mm). Elution with a H_2O/acetonitrile gradient (10 min equilibration with 50% MeCN, 50% to 75% MeCN in 30 min, flow: 20 ml/min) resulted in 5 mg of compound **1** (RT 24.2 min) and 4.1 mg of compound **2** (RT 21.1 min).

Physicochemical properties

Xylarone (**1**): Yellowish oil. – UV (MeOH): λ_{max} (log ε) = 229 (4.42), 331 (3.99) nm. – IR (KBr): ν = 3427, 2960, 1688, 1615, 1558, 1381, 1358, 1249, 1170, 1011, 749 cm^{-1}. – ^1H and ^{13}C NMR: see Table I. – HRMS (ESI, $M+H^+$): m/z = 223.1349 (calcd. for $C_{13}H_{19}O_3$ 223.1334).

8,9-Dehydroxylarone (**2**): Yellowish oil. – UV (MeOH): λ_{max} (log ε) = 253 (4.24), 356 (4.00) nm. – IR (KBr): ν = 3441, 2954, 1683, 1629, 1553, 1383, 1248, 1156, 1012, 957, 807, 747 cm^{-1}. – ^1H and ^{13}C NMR: see Table I. – HRMS (ESI, $M+H^+$): m/z = 221.1188 (calcd. for $C_{13}H_{17}O_3$ 221.1178).

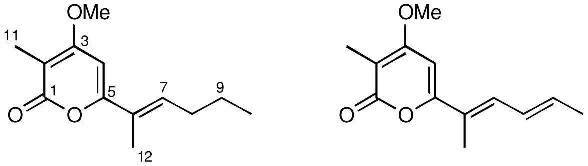
Biological assays

Cytotoxicity was assayed as described previously with slight modifications (Zapf *et al.*, 1995). Colo-320 (DSMZ ACC144), L1210 (ATCC CCI 219) and HL-60 cells (DSMZ ACC3) were grown in RPMI 1640 medium (Invitrogen) and MDA-MB-231 (ATCC HTB-26) and MCF7 cells (ATCC HTB-22) were grown in D-MEM medium (Invitrogen); the media were supplemented with 10% inactivated fetal calf serum (Invitrogen), 65 μg/ml of penicillin G and 100 μg/ml of streptomycin sulphate. Cytotoxic assays were carried out in 96-well-plates with 5×10^4 cells/ml. After 72 h of incubation the IC_{50} value was photometrically determined with Giemsa-staining or with the XTT cell proliferation assay. The minimal inhibitory concentrations against bacteria and fungi were determined as described previously (Anke *et al.*, 1989).

Results and Discussion

Isolation and structural elucidation

Xylarone (**1**) and 8,9-dehydroxylarone (**2**, Fig. 1) were obtained by an activity-guided isolation procedure using L1210 cells as test organisms. The spectroscopic data indicated that they are structurally related, and that they differ in their oxidation status. While the elemental composition of **1** is $C_{13}H_{18}O_3$, as suggested by HRMS experiments, **2** contains two hydrogen atoms less. Inspection of the NMR spectra (^1H and ^{13}C NMR data are presented in Table I) reveals that a propyl group in **1** has been oxidized to a propenyl group in **2**. For xylarone (**1**), the elemental composition shows that the compound has five unsaturations, but the conclusion that the molecule has one carbonyl group and three carbon-carbon double bonds (and thereby contains one ring) was not self-evident because of the polarized carbon-carbon double bonds resulting in unusual carbon shifts. However, starting at the saturated end of the molecule, COSY correlations from 10-H_3 via 9-H_2 and 8-H_2 to 7-H show that the propyl group is attached to a trisubstituted carbon-carbon double bond. 8-H_2 give HMBC correlations to both C-7 and C-6 while 12-H_3 give HMBC correlations to C-5, C-6 and C-7. The chemical shift of C-5 (160.2 ppm) indicates that it is unsaturated and oxygenated, and HMBC correlations from 4-H to C-5 and C-6 show that C-4 (91.6 ppm) is attached to C-5. The pro-



1 **2**

Fig. 1 Structure of xylarone (**1**) and 8,9-dehydroxylarone (**2**).

Table I. ^1H (500 MHz, δ; multiplicity; J) and ^{13}C (125 MHz, δ; multiplicity) NMR spectroscopic data for xylarone (**1**) and 8,9-dehydroxylarone (**2**). The spectra were recorded in $CDCl_3$ and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The coupling constants J are given in Hz. The multiplicities of the carbon signals were determined indirectly from HMQC experiments.

	1		**2**	
Position	δ_H	δ_C	δ_H	δ_C
1	–	165.1; s	–	165.0; s
2	–	102.0; s	–	102.2; s
3	–	165.9; s	–	165.9; s
4	6.11; s	91.6; d	6.15; s	92.1; d
5	–	160.2; s	–	160.0; s
6	–	126.1; s	–	123.2; s
7	6.62; t; 7.4	135.3; d	7.12; d; 11.2	132.2; d
8	2.21; q; 7.4	30.7; t	6.42; dd; 11.2, 15.5	127.4; d
9	1.50; hex.; 7.4	22.2; t	6.10; dd; 6.7, 15.5	136.6; d
10	0.95; t; 7.4	13.9; q	1.89; d; 6.7	19.0; q
11	1.95; s	8.6; q	1.94; s	8.6; q
12	1.89; s	12.4; q	1.97; s	12.4; q
OMe	3.92; s	56.0; q	3.93; s	56.0; q

tons of a methoxy group give strong HMBC correlations to C-3 but also a weak correlation to C-4, and together with the additional HMBC correlations from 4-H to C-2 and C-3 it can be concluded that C-4 is followed by a methoxylated C-3 and thereafter C-2. The remaining methyl group gives HMBC correlations to C-1, C-2 and C-3, and is consequently positioned at C-2. In order to comply with the restrictions imposed by the elemental composition, C-1 must be a carbonyl group in a lactone, connected to C-5. The configuration of the C-6/C-7 double bond was determined by the correlation observed between 8-H_2 and 12-H_3 in the NOESY spectrum. For 8,9-dehydroxylarone (**2**), as mentioned above, the difference is that C-8/C-9 is oxidized to a double bond. The configurations of the double bonds of **2** were determined by the NOESY correlations between 8-H and 10-H_3 as well as 12-H_3, and by the $^1H-^1H$ coupling constant between 8-H and 9-H.

Biological properties

The cytotoxicity of the isolated compounds was moderate. Xylarone (**1**) reduced proliferation of the cells by 50% (IC_{50}) between 40 μg/ml (Colo-320 cells) and 50 μg/ml (L1210 cells). For the other cell lines (MDA-MB-231, MCF7, HL-60) the IC_{50} value exceeded 50 μg/ml. 8,9-Dehydroxylarone (**2**) was slightly more active; IC_{50} values were 25 μg/ml for Colo-320 and L1210 cells, for the other cell lines 50 μg/ml (HL-60) or higher (MDA-MB-231, MCF7). No antibacterial (*Bacillus brevis, B. subtilis, Micrococcus luteus, Enterobacter dissolvens*) and antifungal (*Mucor miehei, Paecilomyces variotii, Penicillium notatum, Nematospora coryli*) activities were detected up to 100 μg/ml of **1**. The same results were obtained for **2** with the exception of *M. luteus* which was inhibited at 100 μg/ml.

α-Pyrones are widespread in nature and have variable biological functions and activities (McGlacken and Fairlamb, 2005). 2-Pyrone derivatives similar to compounds **1** and **2**, phomapyrone A and infectopyrone, were isolated from the stem canker fungus *Leptosphaeria maculans* (Pedras and Chumala, 2005) and nectriapyrone has been reported from different fungi, *e.g. Gyrostroma missouriense* (Nair and Carey, 1975), *Gliocladium vermoesenii* (Avent *et al.*, 1992) or *Pestalotiopsis oenotherae* (Venkatasubbaiah and Van Dyke, 1991). Among the many structurally diverse secondary metabolites described from members of the genus *Xylaria*, this report is the first one on α-pyrone derivatives.

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Anderson J. R. and Edwards R. L. (1985), Metabolites of the higher fungi. Part 22. 2-Butyl-3-methylsuccinic acid and 2-hexyliden-3-methylsuccinic acid from xylariaceous fungi. J. Chem. Soc. Perkin Trans. 1 **7**, 1481–1485.

Anke H., Bergendorff O., and Sterner O. (1989), Assays of the biological activities of guaiane sesquiterpenoids isolated from the fruit bodies of edible *Lactarius* species. Food Chem. Toxicol. **6**, 393–397.

Avent A. G., Hanson J. R., and Truneh A. (1992), Two pyrones from *Gliocladium vermoesenii*. Phytochemistry **31**, 1065–1066.

Breitenbach J. and Kränzlin F. (1984), Pilze der Schweiz, Band 1, Ascomyceten. Verlag Mycologia, Luzern, pp. 276–277.

Dagne E., Gunatilaka A. A. L., Asmellash S., Abate D., Kingston D. G. I., Hoffmann G. A., and Johnson R. K. (1994), Two new cytotoxic cytochalasins from *Xylaria obovata*. Tetrahedron **50**, 5615–5620.

McGlacken G. P. and Fairlamb I. J. S. (2005), 2-Pyrone natural products and mimetics: isolation, characterisation and biological activity. Nat. Prod. Rep. **22**, 369–385.

Nair M. S. R. and Carey S. T. (1975), Metabolites of Pyrenomycetes II: nectriapyrone, an antibiotic monoterpenoid. Tetrahedron Lett. **19**, 1655–1658.

Pedras M. S. C. and Chumala P. B. (2005), Phomapyrones from blackleg causing phytopathogenic fungi: isolation, structure determination, biosyntheses and biological activity. Phytochemistry **66**, 81–87.

Schneider G., Anke H., and Sterner O. (1995), Xylarin, an antifungal *Xylaria* metabolite with an unusual tricyclic uronic acid moiety. Nat. Prod. Lett. **7**, 309–316.

Schneider G., Anke H., and Sterner O. (1996), Xylaramide, a new antifungal compound and other secondary metabolites from *Xylaria longipes*. Z. Naturforsch. **51c**, 802–806.

Venkatasubbaiah P. and Van Dyke C. G. (1991), Phytotoxins produced by *Pestalotiopsis oenotherae*, a pathogen of evening primrose. Phytochemistry **30**, 1471–1474.

Whalley A. J. S. (1996), The xylariaceous way of life. Mycol. Res. **100**, 897–922.

Whalley A. J. S. and Edwards R. L. (1995), Secondary metabolites and systematic arrangement within the Xylariaceae. Can. J. Bot. **73**, 802–810.

Zapf S., Hoßfeld M., Anke H., Velten R., and Steglich W. (1995), Darlucins A and B, new isocyanide antibiotics from *Sphaerellopsis filum* (*Darluca filum*). J. Antibiot. **48**, 36–41.