Isolation and Characterization of Two Verrucarins from *Myrothecium roridum*

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In the course of a screening for compounds inhibiting the growth of two human breast cancer cells lines, two highly cytotoxic compounds were isolated from fermentations of *Myrothecium roridum*. The elucidation of their structures revealed that they are macrocyclic trichothecenes of the verrucarin type, 16-hydroxyverrucarin A (**1**), reported here as a natural product for the first time, and verrucarin X (**2**), a new compound. Both **1** and **2** exhibit moderate antifungal activity and pronounced cytotoxic activity, with IC_{50} values in the nanomolar (**1**) and micromolar (**2**) range. Both compounds preferentially inhibit *in vivo* protein biosynthesis.

Key words: 16-Hydroxyverrucarin A, Verrucarin X, *Myrothecium roridum*, Protein Synthesis Inhibitors

Introduction

The macrocyclic verrucarins (predominantly C27 compounds) and roridins (predominantly C29 compounds) are mycotoxins produced by a variety of fungal genera (Bennett and Klich, 2003). They are of particular concern as contaminants of feed and food causing severe diseases at very low doses. One of their characteristic effects is a quite selective inhibition of protein synthesis (Shifrin and Anderson, 1999). With the exception of 16-hydroxyroridin E, all natural roridins and verrucarins have a methyl group in position 9. In the following we describe the fermentation, isolation, structure determination and biological characterization of the first two natural verrucarins with oxidized C-16. However, 16-hydroxyverrucarin A (**1**) has previously been prepared by selenium dioxide oxidation of verrucarin A, obtained as a minor product in a reaction that mainly produced 8-hydroxyverrucarin A (Jarvis *et al*., 1980). Later the transformation of verrucarin A to **1** was also achieved by the zygomycete *Rhizopus arrhizus* (Pavanasasivam and Jarvis, 1983).

Materials and Methods

General experimental procedures

^{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 probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$ (compound **1**) and CD_3SOCD_3 (compound **2**) and the solvent signals were used as reference (see Tables I and II). 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 (HREI, HRFAB) were recorded with a Jeol SX102 spectrometer, GC-MS analyses were recorded with a micromass GCT instrument while LC-MS experiments were conducted with a LC-MS (HP 1100; APCI, negative mode) and a Micromass Q-TOF MICRO instrument. UV and IR spectra were recorded with a Perkin Elmer 16 instrument and a Bruker IFS 48 spectrometer. The melting points (uncorrected) were determined with a Reichert microscope, and the optical rotations were measured with a Perkin-Elmer 141 polarimeter at 22 °C.

*Producing fungus, fermentation and isolation of compounds **1** and **2***

The producing strain *Myrothecium roridum* 028–98 was isolated from a soil sample. It showed

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all characteristic features of the genus and species (Domsch *et al.*, 1980). The culture is deposited in the culture collection of the Institute of Biotechnology and Drug Research (IBWF), Kaiserslautern, Germany.

For maintenance on agar slants the strain was kept on YMG medium [(g/l): yeast extract (4); malt extract (10); glucose (4); 1.5% agar; pH 5.5].

For the production of 16-hydroxyverrucarin A and verrucarin X *Myrothecium roridum* was grown in 2.5 l of potato dextrose medium [(g/l): dried mashed potatoes (4); glucose (20); pH 5.5] in 5 l Erlenmeyer flasks at 22 °C with agitation (120 rpm). After 42 d when all the glucose had been used up the culture was harvested and the mycelia were separated from the culture fluid by centrifugation ($4000 \times g$). The supernatant was extracted with an equal volume of ethyl acetate and the organic phase dried over Na_2SO_4. After evaporation of the solvent *in vacuo* at 40 °C 165 mg of crude extract were obtained. For preparative HPLC a Jasco Model PU-1586 instrument with a Multiwavelength-Detector MD-910 was used (column: Phenomenex, Luna RP-18, 10 μm, 250×21 mm; gradient: H_2O/acetonitrile 0–100% in 35 min; flow 10 ml/min). **1** was eluted with H_2O/acetonitrile 57:43 (v/v) and **2** with 42:58 (v/v) yielding 14 mg each of **1** and **2**.

*Physicochemical properties of verrucarins **1** and **2***

16-Hydroxyverrucarin A (**1**): UV (MeOH): λ_{max} (log ε) = 260 nm (3.72). – IR (KBr): ν = 3430, 2925, 1715, 1630, 1270, 1190, 1080, 1030, 970, 825 and 615 cm^{-1}. – HRESIMS [M+H$^+$]: measured 519.2241 ($C_{27}H_{35}O_{10}$ requires 519.2230).

Verrucarin X (**2**): UV (MeOH): λ_{max} (log ε) = 259 nm (4.07). – IR (KBr): ν = 3440, 2970, 1715, 1635, 1410, 1270, 1190, 1080, 1030, 970 and 615 cm^{-1}. – HRESIMS [M+H$^+$]: measured 533.2029 ($C_{27}H_{33}O_{11}$ requires 533.2023).

Biological assays

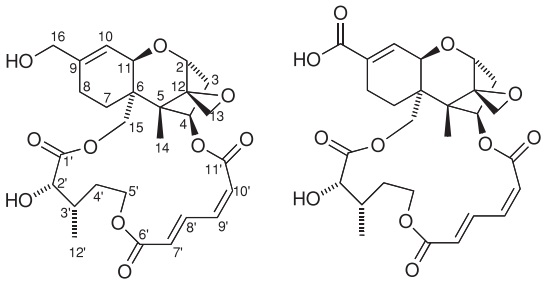
The antimicrobial spectra were determined in an agar plate diffusion test as described previously (Anke *et al.*, 1977). Cytotoxic effects were determined according to Zapf *et al.* (1995) with the following modifications: MCF-7 cells (DSMZ ACC 115, human breast adenocarcinoma), MDA-MB-231 cells (ATCC HTB 26, human breast adenocarcinoma) and HEP-G2 cells (DSMZ ACC 180, human hepatocellular carcinoma) were grown in D-MEM medium (GIBCO, BRL) supplemented with 10% fetal calf serum (FCS) (GIBCO, BRL), 65 μg/ml penicillin G and 100 μg/ml streptomycin sulfate. HT-29 cells (DSMZ ACC 299, human colon adenocarcinoma), COLO-320 cells (DSMZ ACC 144, human colon adenocarcinoma), HL-60 cells (ATCC CCL 240, human promyelocytic leukemia) and Jurkat cells (ATCC TIB 152, human acute T cell leukemia) were cultivated in RPMI medium supplemented with 10% FCS, 65 μg/ml penicillin G and 100 μg/ml streptomycin sulfate. All cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2. To determine the cytotoxic activities of 16-hydroxyverrucarin A and verrucarin X 5×10^4 cells/ml were incubated with or without the test compounds observed under the microscope after 24, 48 and 72 h. For quantification of cytotoxic effects cell lines growing as monolayers were stained with Giemsa, cell lines growing in suspension were treated with XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] after 72 h according to the product information of Roche Diagnostics (Mannheim, Germany).

The influence of the verrucarins on DNA, RNA and protein syntheses in MDA-MB-231 cells was measured as described by Weidler *et al.* (2000). 10^5 cells in 1 ml of D-MEM medium containing 10% fetal calf serum were incubated for 16 h. After preincubation with the test compounds for 30 min at 37 °C 3700 Bq of either [2-^{14}C]-thymidine, [2-^{14}C]-uridine or [1-^{14}C]-leucine (each 1.9–2.0 GBq/mmol) were added. After 24 h the medium was removed and the cells were washed once with ice-cold PBS. Then 0.5 ml of ice-cold TCA were added and the precipitates collected by centrifugation at $14\,000 \times g$ for 10 min at 4 °C. The pellets were resuspended in 1 ml of 5% TCA, centrifuged again, then taken up in 1 ml of scintillation fluid (Quickszint 454) and analyzed in a liquid scintillation counter.

Results and Discussion

Structural elucidation

1 (Fig. 1) has been reported as a semisynthetic product of verrucarin A, but as the structure elucidation was not discussed and the NMR data are not readily available the 1D NMR data are therefore provided here (Tables I and II) along with a short description how the structure was determined. High resolution MS experiments revealed



1 2

Fig. 1. Structures of 16-hydroxyverrucarin A (**1**) and verrucarin X (**2**).

that the elemental composition of **1** is $C_{27}H_{34}O_{10}$, and that was consistent with the presence of 27 peaks in the decoupled ^{13}C NMR spectrum and signals integrating for 32 protons in the ^{1}H NMR spectrum. **1** consequently has 11 unsaturations, and since NMR data suggest that the molecule contains three carbon-carbon double bonds and three carbonyl groups, it should be pentacyclic. The basic trichothecene skeleton was deduced from the following COSY and HMBC correlations: The HMBC correlations from 2-H to C-3, C-11 and C-12 as well as the COSY correlations from 2-H to 3-H_2; the HMBC correlations from 3-H_2 to C-2 and C-4 as well as the COSY correlations from 3-H_2 to 2-H and 4-H; the HMBC correlations from 13-H_2 to C-2, C-5 and C-12, from 14-H_3 to C-4, C-5, C-6 and C-12, and from 15-H_2 to C-5, C-6, C-7 and C-11; the COSY correlation between 7-H_2 and 8-H_2, the HMBC correlations from 16-H_2 to C-8, C-9 and C-10, the COSY correlations between 10-H and 11-H, and the HMBC correlation from 11-H to C-2. In addition, the chemical shifts for 13-H_2, C-12 and C-13 are typical for an epoxide moiety, as are the $^{1}H/^{13}C$ coupling constant of 13-H_2/C-13 of 177 Hz. The fifth

Table I. ^{1}H NMR (500 MHz) data (δ; multiplicity; J) for 16-hydroxyverrucarin A (**1**) and verrucarin X (**2**). The spectrum of **1** was recorded in $CDCl_3$ while the spectrum of **2** was recorded in CD_3SOCD_3, and the solvent signals (7.26 and 2.50 ppm, respectively) were used as reference. The coupling constants J are given in Hz.

H	**1**	**2**
2	3.88; d; 5.4	3.77; d; 5.0
3α	2.51; dd; 8.2, 15.4	2.47; dd; 8.1, 15.4
3β	2.26; ddd; 3.7, 5.4, 15.4	2.01; ddd; 3.8, 5.0, 15.4
4	5.83; dd; 3.7, 8.2	5.85; dd; 3.8, 8.1
7α	1.76; m	1.63; m
7β	1.91; m	1.76; m
8α	1.93; m	1.86; m
8β	2.08; m	2.31; m
10	5.73; d; 5.0	6.49; d; 4.0
11	3.64; d; 5.0	3.91; m
13a	3.12; d; 3.9	3.04; 4.0
13b	2.82; d; 3.9	2.79; d; 4.0
14	0.86; s	0.75; s
15a	4.78; d; 12.1	4.27; d; 21.5
15b	4.24; d; 12.1	4.10; d; 12.5
16a	4.11; d; 14.1	–
16b	4.07; d; 14.1	–
2′	4.15; d; 1.7	4.06; s
3′	2.37; m	2.19; m
4′a	1.93; m	1.79; m
4′b	1.78; m	1.63; m
5′a	4.52; ddd; 2.5, 5.3, 11.4	4.33; 3.0, 5.9, 11.2
5′b	3.98; ddd; 3.1, 11.4, 12.1	3.92; m
7′	6.06; d; 15.7	6.22; d; 15.6
8′	8.04; dd; 11.7, 15.7	7.84; dd; 11.7, 15.6
9′	6.68; dd; 10.9, 11.7	6.86; dd; 11.1, 11.7
10′	6.16; d; 10.9	6.32; d; 11.1
12′	0.88; d; 6.8	0.78; d; 6.8

Table II. ^{13}C NMR (125 MHz) data (δ; multiplicity) for 16-hydroxyverrucarin A (**1**) and verrucarin X (**2**). The spectrum of **1** was recorded in $CDCl_3$ while the spectrum of **2** was recorded in CD_3SOCD_3, and the solvent signals (77.00 and 39.51 ppm, respectively) were used as reference. The multiplicities of the carbon signals were determined indirectly from HMQC experiments.

C	**1**	**2**
2	79.0; d	78.2; d
3	34.8; t	34.5; t
4	75.4; d	75.7; d
5	49.5; s	48.9; s
6	44.7; s	43.6; s
7	19.6; t	19.3; t
8	22.9; t	22.0; t
9	143.8; s	135.9; s
10	117.6; d	130.8; d
11	66.3; d	65.2; d
12	65.1; s	65.0; s
13	47.8; t	46.8; t
14	7.3; q	7.0; q
15	63.3; t	61.5; t
16	65.8; t	167.9; s
1′	174.7; s	173.5; s
2′	74.2; d	72.9; d
3′	33.2; d	32.4; d
4′	32.2; t	31.9; t
5′	61.1; t	61.0; t
6′	165.4; s	164.8; s
7′	127.5; d	127.3; d
8′	138.8; d	138.5; d
9′	139.0; d	138.1; d
10′	125.7; d	126.3; d
11′	166.1; s	165.6; s
12	10.0; q	10.5; q

ring starts at C-15 as indicated by the HMBC correlations from 15-H_2 to C-1′, which has an α-hydroxy group as indicated by the chemical shifts of 2′-H/C-2′ as well as by the HMBC correlation from 2′-H to C-1′. The spin system from C-2′ to C-5′, which is oxygenated according to its chemical shift, was evident, and the fact that the oxygen atom is acylated is shown by the chemical shift of 5′-H_2 as well as by the HMBC correlation of 5′-H_2 to C-6′. The link between the two carbonyls C-6′ and C-11′ was established by the COSY correlations, and the configurations of the two double bonds were determined by the $^1H-^1H$ coupling constants. Finally, the last ring is closed by the HMBC correlation from 4-H to C-11′.

The relative stereostructure of **1** was determined by the correlations observed in the NOESY spectrum. The correlations between 11-H and 3-Hα, 4-H as well as 15-H_2, between 2-H and 13-Ha, between 13-Hb and 7-H_2, and between 14-H_3 and 7-Hα as well as 13-Hb show that the trichothecene part has the expected relative configuration. The macrocycle is folded in the way that 8′-H comes near 14-H_3 and 3′-H, according to the correlations observed in the NOESY spectrum, but as there is no correlation between 3′-H and 14-H_3, 8′-H should be placed in space between 3′-H and 14-H_3. Correlations between 15-Ha and 11-H, 15-Hb and 4-H, as well as between 2′-H and 14-H_3 define the folding of the C-1′ ester moiety, and a Dreiding model of **1** with the configuration of C-2′ and C-3′ as shown in Fig. 1 with the above discussed restrictions explains the strong NOESY correlations observed between 12′-H_3 and 5′-Hb, 2′-H and 4′-Ha, and 3′-H and 4′-Hb. In addition, the small $^1H-^1H$ coupling constant between 2′-H and 3′-H (1.7 Hz) is reasonable according to the dihedral angle between the protons in the suitable conformation.

The structure of verrucarin X (**2**) (Fig. 1) was determined from the corresponding data, with the difference that the elemental composition of **2** is $C_{27}H_{32}O_{11}$ and the $-CH_2OH$ group was exchanged for a $-COOH$ group. The position of this carboxyl group could be established by the HMBC correlations from 8-H_2 and 10-H to C-16.

Biological properties

In the plate diffusion assay no antibacterial activities could be detected for verrucarins **1** and **2** at contents up to 100 μg/disc. The effects on fungal growth were moderate for *Nematospora coryli* and weak for *Mucor miehei*. The results are summarized in Table III and are comparable with the data previously reported (Wagenaar and Clardy, 2001).

Table III. Antibacterial and antifungal activities of verrucarins **1** and **2** in the agar plate diffusion assay at 100 μg/disc.

	Inhibition zone [mm]	
Organism	**1**	**2**
Bacillus subtilis	–	–
Bacillus brevis	–	–
Enterobacter dissolvens	–	–
Micrococcus luteus	–	–
Penicillium notatum	7	–
Paecilomyces variotii	12	–
Mucor miehei	14	7
Nematospora coryli	23	20

As is the case for other verrucarins and roridins **1** and **2** exhibit strong cytotoxic activities against all tested human cancer cell lines. The activity of **1** surpassed the activity of **2** in all cases by one or two orders of magnitude. The most sensitive cell lines were COLO-320, HL-60 and Jurkat with IC_{50} values in the low nanomolar range for **1** and in a high nanomolar range for **2** (Table IV) which corresponds to data published for other verrucar-

Table IV. Cytotoxic activities of verrucarins **1** and **2**.

Cell line	**1**		**2**	
	IC_{50} [μg/ml]	IC_{50} [μM]	IC_{50} [μg/ml]	IC_{50} [μM]
MDA-MB-231	0.021	0.040	2.9	5.4
MCF-7	0.007	0.014	0.81	1.5
HT-29	0.018	0.035	0.23	0.43
COLO-320	0.004	0.008	0.10	0.19
HEP-G2	0.018	0.035	3.8	7.1
HL-60	0.005	0.010	0.49	0.9
Jurkat	0.002	0.004	0.48	0.9

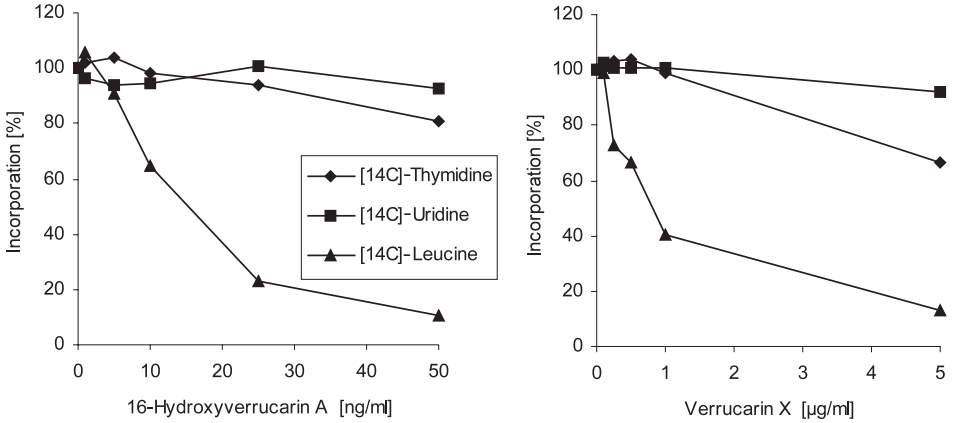


Fig. 2. Incorporation of radiolabelled precursors in macromolecules of MDA-MB-231 cells. The influence of both verrucarins on synthesis of cellular DNA, RNA and proteins was detected in MDA-MB-231 cells by measuring the incorporation of [^{14}C]-thymidine, [^{14}C]-uridine and [^{14}C]-leucine in TCA-insoluble fractions (Weidler *et al*., 2000). Controls (100%): Thymidine 29.6 Bq, uridine 81.5 Bq, leucine 65.2 Bq.

ins and roridins (Wagenaar and Clardy, 2001; Abbas *et al*., 2002).

Trichothecenes are potent inhibitors of eukaryotic protein synthesis interfering with either the initiation or elongation process by binding to the peptidyl transferase catalytic center. Like anisomycin trichothecenes induce a ribotoxic stress, activate cJun *N*-terminal kinase/p38 mitogen activated protein kinases and are strong inducers of apoptosis (Shifrin and Anderson, 1999; Zhou *et al*., 2003).

The influence of verrucarins **1** and **2** on cellular DNA, RNA and protein syntheses of MDA-MB-231 cells is shown in Fig. 2. Inhibition of protein synthesis is pronounced and selective with IC_{50} values of 16 ng/ml for **1** and 0.8 μg/ml for **2**.

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Abbas H. K., Johnson B. B., Shier W. T., Tak H., Jarvis B. B., and Boyette C. D. (2002), Phytotoxicity and mammalian cytotoxicity of macrocyclic trichothecene mycotoxins from *Myrothecium verrucaria*. Phytochemistry **59**, 309–313.

Anke T., Oberwinkler F., Steglich W., and Schramm G. (1977), The strobilurins – new antifungal antibiotics from basidiomycete *Strobilurus tenacellus* (PERS. ex FR.) SING. J. Antibiot. **30**, 806–810.

Bennett J. W. and Klich M. (2003), Mycotoxins. Clin. Microbiol. Rev. **16**, 497–516.

Domsch K. H., Gams W., and Anderson T. (1980), Compendium of Soil Fungi. Academic Press, New York.

Jarvis B. B., Stahly G. P., and Pavanasasivam G. (1980), Antileukemic compounds derived from chemical modification of macrocyclic trichothecenes. 1. Derivatives of verrucarin A. J. Med. Chem. **23**, 1054–1058.

Pavanasasivam G. and Jarvis B. B. (1983), Microbial transformation of macrocyclic trichothecenes. Appl. Environm. Microbiol. **46**, 480–483.

Shifrin V. I. and Anderson P. (1999), Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun *N*-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. J. Biol. Chem. **274**, 13985–13992.

Wagenaar M. M. and Clardy J. (2001), Two new roridins isolated from *Myrothecium* sp. J. Antibiot. (Tokyo) **54**, 517–520.

Weidler M., Rether J., Anke T., and Erkel G. (2000), Inhibition of interleukin-6 signaling by galiellalactone. FEBS Lett. **484**, 1–6.

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. (Tokyo) **48**, 36–41.

Zhou H. R., Lau A. S., and Pestka J. J. (2003), Role of double-stranded RNA-activated protein kinase R (PKR) in deoxynivalenol-induced ribotoxic stress. response. Toxicol. Sci. **74**, 335–344.