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Laccaridiones A and B, New Protease Inhibitors from *Laccaria amethystea*

Sir:

There is growing interest in new inhibitors of proteases and metalloproteases due to their role in various human diseases.

In a screening for new inhibitors of proteases as lead structures we disclosed recently the basidiomycete strain of *Laccaria amethystea* as the producer of active compounds. Here we report the isolation and structure determination of laccaridiones A (**1**) and B (**2**) (Fig. 1) as new inhibitors of trypsin, papain, thermolysin, collagenase and zinc-protease.

The strain was isolated from the stem of fruiting body of *Laccaria amethystea* in the *Fagus sylvatica*-forest near Jena (Thuringia, Germany). At the beginning the flat mycelium shows a cream to greyish colour with a violet colour-component, later on it turns to grey-violet until violet-brown with a somewhat bright violet-grey air-mycelium staining the agar intensively from brown-violet to violet-black. The hyphae are hyaline, about 2 μm thick, later thicker and septated, about 6 μm in diameter, finally some hyphae are inflated with thick short cells (16/10 μm). Air mycelium shows guarted and branched structures and in some cases also inflated hyphae tips similar to chlamydospores. The cell content is violet and it changes later to brown-violet.

These properties suggest that the strain is a representative of *Laccaria amethystea*[1].

The producer strain was cultivated as surface culture at 24°C in 500 ml Erlenmeyer bottles containing 100 ml medium composed as follows (g/liter): malt extract 20, glucose 10, yeast extract 1, pH 6.0. Each bottle was inoculated with a 2 cm^2 area of 21 days agar culture. The cultivation time was three weeks at 24°C.

1 and **2** were extracted from 30 liters of the surface culture of *Laccaria amethystea* with 20 liters of ethyl acetate, which was subsequently dried (Na_2SO_4) and evaporated to obtain 1.5 g of a deep purple oil. This was chromatographed subsequently on silica gel 60 (Merck, 0.063~0.1 mm, column 600×30 mm). Sequential elution occurred with 400 ml portions of *n*-hexane, *n*-hexane-chloroform (7:3, 1:1, 1:3, 0:1, v/v). Fractions of similar composition as determined by TLC analysis were pooled

Fig. 1. Structures of laccaridione A (**1**) and laccaridione B (**2**).

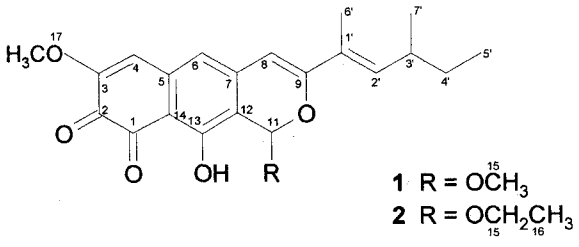


Table 1. Physico-chemical properties of laccaridiones A (**1**) and B (**2**).

	(1)	(2)
Appearance	deeply reddish solid	deeply reddish solid
Melting point[a]	164 - 165 °C	178 - 179 °C
Chemical formula	$C_{22}H_{24}O_6$	$C_{23}H_{26}O_6$
HREI-MS *m/z*	384.1583 $[M]^+$ calcd. 384.1573	398.1712 $[M]^+$ calcd. 398.1729
UV (λ_{max}; nm) in parantheses ε; (cm^2/Mol, in MeOH)[b]	239 (19300), 298 (17000), 481 (12800)	239 (19000), 299 (16500), 480 (12100)
Rf on TLC ($CHCl_3$: MeOH, 99.5:0.5, v/v)	0.8	0.7

[a] Büchi Melting Point B 540

[b] Beckmann DU 600

Table 2. ^{1}H and ^{13}C assignments of laccaridiones A (**1**) and B (**2**) (in $CDCl_3$, chemical shifts in ppm, multiplicity in parentheses (s: singlet, d: doublet, t: triplet, m: multiplet), coupling constants in Hz).

Position	1		2	
	δ_C	δ_H	δ_C	δ_H
1	178.9	-	178.8	-
2	176.9	-	177.0	-
3	152.0	-	152.0	-
4	113.7	6.3 (s)	113.7	6.3 (s)
5	157.0	-	158.0	-
6	118.6	6.5 (s)	118.7	6.5 (s)
7	144.2	-	142.1	-
8	99.9	6.0 (s)	99.9	6.0 (s)
9	161.8	-	157.8	-
10	-	-	-	-
11	94.6	6.3 (s)	93.4	6.4 (s)
12	112.8	-	113.7	-
13	164.0	-	164.0	-
14	110.9	-	111.0	-
15	55.4	3.6 (d; 7.2)	64.1	3.8; 3.9 (m)
16	-	-	15.2	1.3 (t; 7.2)
17	55.7	3.8 (s)	5.7	3.8 (s)
1'	127.1	-	127.2	-
2'	140.8	6.3 (dd; 8.1, 1.5)	140.7	6.3, (dd; 8.0, 1.2)
3'	34.8	2.4 (m)	34.8	2.5)m)
4'	30.1	1.4; 1.5 (m)	30.1	1.4, 1.5 (m)
5'	11.9	0.8 (t; 7.1)	11.9	0.8 (t; 7.0)
6'	12.8	1.9 (s)	12.8	1.9 (s)
7'	20.2	1.0 (d; 6.8)	20.2	1.0 (d; 6.9)
13-OH	-	12.6 (s)	-	12.6 (s)

to yield 50 mg of **1** and **2** as a brownish mass. Final purification was carried out by chromatography on Sephadex LH-20 (column 400×20 mm; eluent dichloromethane - hexane (4 : 1, v/v)). Yield: 13 mg of laccaridione A (**1**) and 17 mg laccaridione B (**2**).

Important physico-chemical properties of **1** and **2** are shown in Table 1. The presence of an *ortho*-benzoquinone ring system was suggested by the UV spectra of **1** and **2** (λ_{max} 299 and 481 nm; shift to 392 and 542 nm upon the addition of NaOH)[2,3]. The molecular weight and the chemical formula of the novel metabolites were determined from the HREI mass spectra (double-focusing mass spectrometer AMD 402; Intectra Harpstedt, Germany) (Table 1). The positive ion electrospray ionization mass spectra showed *m/z* 385.1 $[M+H]^+$ of **1** and *m/z* 399.3 $[M+H]^+$ of **2** (triple quadrupole mass spectrometer Quattro 400; VG Biotech; Altrincham, U.K.).

Information about the length of the side chain was furnished by the EI-MS fragmentation pattern. Thus the loss of a C_5H_9 moiety *m/z* 69 suggested a 1,3-dimethylpentyl group and its binding to an oxygen-substituted vinylic carbon.

The structures of compounds **1** and **2** were determined by one- and two-dimensional NMR experiments (^{1}H, ^{13}C, DEPT, COSY, HMBC, HSQC, NOESY; Table 2; Bruker Avance DRX 500, Germany).

The ^{1}H NMR spectra displayed two aromatic singlet protons, aliphatic doublet- and triplet methyl groups, signals of two methoxyl groups (in **1**) and two methylene and methine protons. The latter CH–CH_3 and CH_2–CH_3

groups were attributable to the aliphatic side-chain. **2** was distinguishable by an oxymethylene signal instead of a methoxyl group and an additional triplet proton methyl group. ^{13}C NMR data confirmed the presence of twenty-two and twenty-three carbons respectively. Distinguishable were two carbonyl groups and several sp^2- and sp^3-hybridized carbon atoms[4].

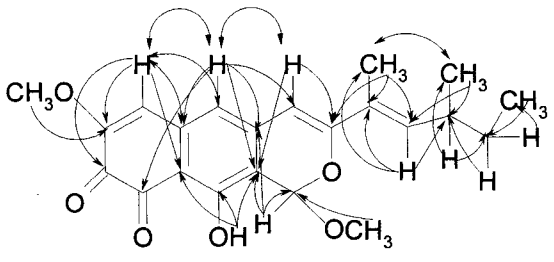
As shown in Fig. 2 every carbon atom of **1** and **2** was assignable by the C,H long-range correlations observed in the HMBC spectra[5]. The presence of an *ortho*-quinone moiety was confirmed by the observed C,H long-range correlations between H-4 and C-2 ($^4J_{C,H}$; weak).

The stereochemistry of the side chain double bond was assigned as *E* (Fig. 1) due to the strong NOE effect between H-6′ and H-7′ in the NOESY spectra of **1** and **2** at the one side, and the missing NOE effect between H-2′ and H-6′ at the other. Further NOE effects between H-4 and H-6 and H-6 and H-8 in the ringsystem supported unambiguously the proposed structure in combination with long-range correlations of the HMBC spectra.

Metabolites **1** and **2** inhibited a series of proteases such as trypsin, papain, thermolysin, collagenase (all Sigma) and zinc-protease from *Bacillus subtilis*. The mode of inhibition of proteases by **1** and **2** remains to be clarified. Though *p*-quinones and hydroxyquinones have been reported as nonpeptide inhibitors of peptide-cleaving enzymes[6,7], no information is available on *o*-quinones, especially naphthopyran-8,9-diones. The IC_{50} values are shown in Table 3.

Laccaridione B (**2**) displayed strong antiproliferative effect on the murine fibroblast-cell line L-929 (IC_{50}= 2.4 μg/ml) and the human leukemia cell line K-562 (IC_{50}= 1.8 μg/ml). However, the cytotoxic effect on HeLa was moderated with a IC_{50} value of 13.9 μg/ml.

Fig. 2. Instructive C, H long-range couplings (HMBC spectrum (⟶) and NOE's in the NOESY spectrum (⟷) of **1**).



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ALBRECHT BERG*,†
KATHRIN REIBER†
HEINRICH DÖRFELT††
GRIT WALTHER††
BRIGITTE SCHLEGEL†
UDO GRÄFE†

†Hans-Knöll-Institute for Natural Products Research, Beutenbergstr. 11, D-07745 Jena, Germany
††Institute of Ecology and Environment, University of Jena, Dornburgerstr. 159, D-07743 Jena, Germany

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Table 3. Inhibitory activity of **1** and **2** against proteases as measured with labelled casein substrate[8].

	IC_{50} (μg/ml)				
	trypsin	**papain**	**thermolysin**	**collagenase**	**zinc-protease**
1	14.7	2.5	18.8	7.2	18.2
2	10.9	5.1	8.4	5.7	3.0

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