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Agrocybolacton, a New Bioactive Metabolite from *Agrocybe* sp. HKI 0259

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In the course of our continuing search for new bioactive fungal products we disclosed recently the strain *Agrocybe* sp. HKI 0259 as the producer of a new antibacterial compound. The strain *Agrocybe* sp. HKI 0259 was isolated from the basidiospores of *Agrocybe* spec. (Pediadeae) in a deciduous forest with *Lithocarpus pachycarpa*, *Archidendron clypearia*, *Eurya nidita* and *Elaeocarpus varunua* in Bach Ma (Vietnam).

The pileus of the basidiomata is 4.4 cm in diameter, beige-brown, in the middle brown and lamellae are greyish to brownish with slightly violet colour, in the middle 5 mm broad. On the edge of the lamellae cheilocystidia are located. The stipe is 8.5 cm long, white, hollow, fragile, without veil and on the base with rhizomorphs.

Spore print is tobaccobrown, spores 9~12.5/6~6.5 μm with thick double wall and conspicuous germ pore. The mycel is white with hyphae 2~2.5 μm in diameter, septate. The septa contain clamps.[1)]

The producer strain *Agrocybe* sp. was cultivated as surface cultures at 25°C in 500 ml Erlenmeyer bottles containing 100 ml medium composed as follows (g/liter): malt extract 20, glucose 10, yeast extract 1, $(NH_4)_2SO_4$ 5, pH 6.0. Each bottle was inoculated with 1 cm^2 area of a 20 days agar culture. After 20 days of cultivation at 25°C, the mycelium cake was harvested from 20 liters of culture and extracted twice with 5 liters of ethyl acetate. The culture broth was extracted threetimes with 10 liters of ethyl acetate. The combined extracts were dried and evaporated. The residue (2.8 g) was subjected to silica gel chromatography (silica gel 60, Merck, 0.063~0.1 mm, column 4×60 cm), using stepwise $CHCl_3$, and $CHCl_3$ - MeOH (9 : 1, 8 : 2, 1 : 1, v/v) as eluents. The active component was isolated on the basis of a biological assay. 1 mg of each fraction were solved in 1 ml methanol, 50 μl of these solutions were transfered on a *Bacillus subtilis* plate. The active fractions were combined and detected also by monitoring the mass spectrum, which identified the active substance as *m/z* 265 during ESI-MS.

Final purification was achieved by preparative HPLC (Spherisorb ODS-2 RP_{18}, 5 μm (Promochem), 250×25 mm, acetonitrile/H_2O; 83 : 17 v/v, 10 ml/minute, UV-detection 210 nm). Yield 17 mg of agrocybolacton (**1**).

The IR spectrum of **1** showed 1751 cm^{-1} in accord with the presence of a carbonyl group. Supporting evidence was furnished by the UV-VIS spectrum showing λ_{max} 207 nm. Optical rotation $[\alpha]_D$=42.8° revealed the chiral nature of **1**.

The HREI-MS *m/z* 264.1384 (M^+; calcd. 264.1406 for $C_{15}H_{20}O_4$) disclosed readily the molecular weight and the chemical formula of **1** (double-focussing mass spectrometer MAT 95XL, Finnigan, Bremen, Germany). The latter suggested the presence of six double bonds and/or rings in the molecule. Conclusive evidence for the chemical structure (relative stereochemistry) of **1** was furnished by 1D and 2D NMR spectroscopy (^1H, ^{13}C, DEPT, COSY, HMQC, HSQC).

The ^1H NMR spectrum displayed two singlet proton signals (0.92 ppm, 1.14 ppm) attributable to methyl groups. Moreover, one olefinic proton signal (5.91 ppm, s, broad) was visible. According to the $^1H,^1H$-COSY spectrum this proton was coupled with H_a-8 and H_b-8. Due to their chemical downfield shift four protons (3.75 ppm (H-3), 5.98 ppm (H-13), 4.39 ppm (H_A-13), 4.45 ppm (H_B-13)) were attributable to oxygen-bonded carbons.

In the ^{13}C NMR spectrum one ester carbonyl signal (174.2 ppm), two olefinic carbons (124.4 ppm (d), 132.1 ppm (s)), and two oxygen-bonded carbons (71.7 ppm (C-13), 65.1 ppm (C-3)) were observed. The carbon signal at 103.2 ppm (d) was assignable to a cyclic acetal structure[2)] (Fig. 1). The multiplicity of the carbon atoms was readily proposed by the DEPT spectrum.

The sequences of coupling protons (H-3/H-4/H-5, H-9/H-10/H-11, H-12/H-13) were afforded by the $^1H,^1H$-COSY spectra. For the assignment of the other constituting protons and carbons, and the relative stereochemistry the C,H long-range coupled NMR spectra (HMBC) and the observable NOESY connectivities were particularly helpful. Especially the C,H long-range couplings of H-12 with C-1, C-11 and C-13, of H-7 with H-1, H-3 and H-11

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Table 1. Physico-chemical properties of **1**.

	1
Appearance	White solid
Melting point[a]	136 - 138 C °
Molecular weight (HREI-MS)	*m/z* 264.1384 (M^+; calcd. 264.1406)
Formula	$C_{15}H_{20}O_4$
UV-VIS[b] (λ_{max}, nm), (ε), in MeOH	207 (16400)
IR[c] (cm^{-1}), film	3400, 2920, 2861, 1751
R_f on TLC (silica gel 60)[d]	0.6
$[\alpha]_D^{20}$ (c 0.3, MeOH)[e]	42.8 °
R_t (min) on HPLC[f]	10.2

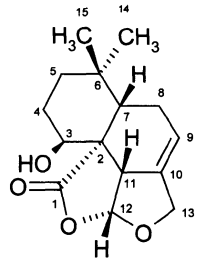
[a] Büchi Melting Point Apparatus B540 Konstanz, Germany
[b] SPECORD 200 Carl Zeiss Jena, Germany
[c] Satellite FTIR Mattson Madison, USA
[d] $CHCl_3$-MeOH (9:1, v/v)
[e] Propol Dr. Kernchen Seelze, Germany
[f] acetonitrile/0,1 % TFA

Table 2. Assignments of ^1H and ^{13}C NMR spectra of **1** (500 MHz; in $CDCl_3$, chemical shifts in ppm; coupling constants in Hz, TMS as internal standard).

	1		
Position	δ_C	δ_H	**$^1H,^1H$-COSY**
1	174.2 (s)	-	-
2	54.6 (s)	-	-
3	65.1 (d)	3.75 br; 5.40 br (OH)	H-4
4	25.5 (t)	1.58 dd; 13.9; 1.5; 1.75 ddd; 13.9; 1.5; 13.5	H-3, H-5
5	33.6 (t)	1.15 m; 1.80 dd; 12.0; 13.5	H-4
6	31.9 (s)	-	-
7	38.3 (d)	1.92 dd; 11.9; 5.2	H-8
8	23.8 (t)	2.00 t,br; 2.22 d,br; 17.1	H-7, H-9
9	124.4 (d)	5.91 s,br	H-8
10	132.1 (s)	-	-
11	47.8 (d)	3.38 br	H-12
12	103.2 (d)	5.98 d; 5.1	H-11
13	71.7 (t)	4.39 dd; 11.0 br; 4.45 dd; 11.0 br	-
14	21.9 (q)	1.14 s	-
15	32.4 (q)	0.92 s	-

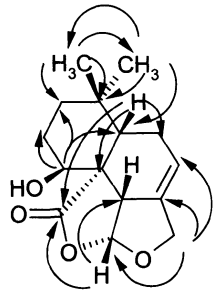
Abbreviations: s: singlet, d: doublet, t: triplet, q: quartet, br: broad, multiplicity in parentheses, (Bruker Avance DRX 500).

Fig. 1. Structure of agrocybolacton (**1**).



1

Fig. 2. Instructive C,H long-range couplings in the HMBC spectrum of **1**.



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confirmed the chemical constitution of **1** (Fig. 2). The relative stereochemistry of **1** was settled on the basis of the observable NOE correlations between H-11/H-12 and H-11/H-7, on the one side, and the missing NOE correlation between H-3/H-7 and H-3/H-11 on the other, respectively. Metabolite **1** thus appears as a novel fungal metabolite possessing an unusual tetracyclic ring system.

Agrocybolacton (**1**) displays moderat antibacterial activity against Gram-positive bacteria such as *Bacillus subtilis* ATCC 6633 and *Mycobacteria smegmatis* SG987 in concentration >50 μg/ml. However, no activity of the same concentration was found against fungi and yeasts[3].

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