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Hexacyclinol, a New Antiproliferative Metabolite of *Panus rudis* HKI 0254

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During our continuing screening for new bioactive metabolites from fungi we discovered recently hexacyclinol (**1**) as a novel, unusual, oligocyclic metabolite in cultures of the fungal strain *Panus rudis* HKI 0254. The strain was isolated from basidiospores of this fungus found on dead betula woods collected near Irkutsk (Sibiria, specimen herb. H. DÖRFELT, Sibiria 299). *Panus rudis* is of widespread occurrence throughout the world in many different ecotypes and has been reported to produce bioactive secondary metabolites[1,2]. The basidiomata of *Panus rudis* from the Sibirian source are growing singly or basal confluent to little tufts, excentric to central, stipitate, infundibuliform, with hazel-brown, strong tomentous pilei until 4.5 cm in diameter; pilei and stipes with hairs until 2.5 mm, basidiospores approx. 5×1.5 μm. *Panus rudis* HKI 0254 shows in cultures a white to light-brownish mycelium, tomentous with clamped connections.

For cultivations a small piece of a mature slant culture of the strain HKI 0254 grown on malt extract agar (malt extract 4%, yeast extract 0.4%, agar 1.5%, deionized water ad 1 liter, pH 6.0) was used to inoculate 1000 ml Erlenmeyer bottles containing 250 ml of the producing medium consisting of glucose 1%, sucrose 1%, corn starch 1%, yeast extract 0.8%, casein peptone 0.1%, soybean meal 0.5%, $(NH_4)_2HPO_4$ 0.05%, $CaCO_3$ 0.03%, $(NH_4)SO_4$ 0.5%. The surface cultures were incubated for 21 days at 23°C.

The fermentation broth (15 liters) was extracted twice overnight by stirring with ethyl acetate (1 : 1). The combined extracts were dried and evaporated to dryness (yield: 5.6 g). The residue was chromatographed on Sephadex LH-20 (MeOH), samples of eluted fractions were spotted on TLC and stained by vanillin/H_2SO_4. Fractions staining blueish-greenish were pooled and separated subsequently by column chromatography on silica gel 60 (Merck 0.063~0.1 mm; column 40 cm×4 cm; elution by $CHCl_3$/MeOH 95 : 5), and TLC on silica gel 60 aluminium sheets (Merck; $CHCl_3$/MeOH; 95 : 5, v/v) to yield 25 mg **1** as a colorless solid in addition to the bulk of recurrent metabolites such as pyrenocin-A, pyrenocin-B, deoxy-phomalone, 2,2-dimethyl-3-hydroxy-6-methoxy-chromanone, and 7-desoxy-panepoxydol[3,4]. The physico-chemical properties of **1** are shown in Table 1.

Structure elucidation of **1** (Fig. 1a) was done using optical spectroscopy, mass spectrometry, 1D and 2D NMR spectroscopy spectroscopy (^1H, ^{13}C, DEPT, COSY, HMQC, HMBC, NOESY). Absorbances at 1625, 1698, 1700 and 3415 cm^{-1} (FTIR instrument Sattelite, Mattson, USA) suggested the presence of double bonds, carbonyl and hydroxyl groups. In the UV spectrum (Specord 2000, Analytik Jena, Germany) maxima were visible at 210 and 249 nm.

Positive ion ESI-MS (triple quadrupole instrument Quattro, VG Biotech, Altrincham, Emgland; recorded in presence of ammonium acetate) showed *m/z* 417.2 ($[M+H]^+$) and *m/z* 434.3 ($[M+NH_4]^+$). In the HREI-MS (Finnigan MAT 95XL) *m/z* 416.1867 (6%, calcd. 416.1835 for $C_{23}H_{28}O_7$) was visible as the molecular ion. Diagnostic fragments such as *m/z* 384.1573 (15%, $[M-CH_3OH]^+$; calcd. 384.1572 for $C_{22}H_{24}O_6$) and *m/z* 369.1343 (100%; $[M-CH_3OH, -CH_3]^+$, calcd. 369.1348 for $C_{21}H_{21}O_6$) confirmed $C_{23}H_{28}O_7$ as the elemental composition. The chemical formula of **1** attested to the occurrence of 10 double bonds or rings in the molecule. The proton NMR spectrum (Bruker Avance DRX 500; Table 2) displayed signals of four methyl (1.78 ppm, 1.78 ppm, 1.28 ppm, 1.15 ppm), one methoxyl (3.01 ppm), twelve methine and

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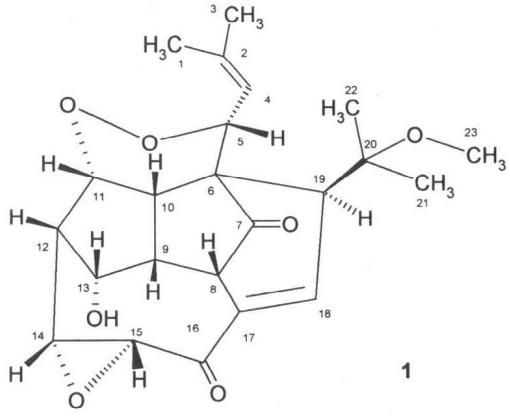
Table 1. Physico-chemical properties of **1**.

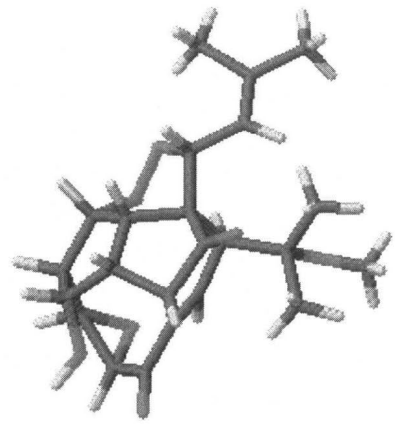
	1
Appearance	Colorless solid
M.P.	172-173 °C
Molecular weight (HREI-MS) M^+	m/z 416.1867 (found) m/z 416.1835 (calcd.)
Formula	$C_{23}H_{28}O_7$
IR (λ_{max}, cm^{-1})	910, 980, 995, 1130, 1009, 1144, 1215, 1385, 1446, 1625, 1698, 1700, 2936, 2980, 3015, 3415
UV (λ_{max}, nm); (ε (cm^2/mol))	205 (6.25 .10^6), 249 (2.1 . 10^6)
$[\alpha]_D^{22}$ (4.03 mg/ml, 0.5 cm)	+ 130.5 °
R_f (TLC, silica gel 60, aluminium sheets, $CHCl_3$/MeOH 9:1, v/v)	0.85
HPLC (RP_{18} 4 mm x 250 mm, gradient 95 % H_2O to 95 % MeCN, 1 ml/min), R_t(min)	12.3

Fig. 1a. Structure (relative stereochemistry) of hexacyclinol (**1**).



1

Fig. 1b. Calculated 3D structure (ACD lab programme).



1

one hydroxyl group (OH-13; 2.56 ppm, br). Due to their chemical shift pattern three of the methine protons were attributable to oxygen-bonded carbons. The sequence of protons in **1** was proposed by the $^1H,^1H$ coupling pattern as shown in the $^1H,^1H$-COSY spectrum (Table 2). The ^{13}C NMR spectrum disclosed the presence of 23 carbon atoms in the molecule. The DEPT spectrum suggested the occurrence of two keto groups (202.9 ppm (C-7), 192.8 ppm (C-16)), two double bonds (142.5 ppm (s); 120.7 ppm (d); 132.5 ppm (s); 139.6 (d)), two quaternary carbons (60.5 ppm and 77.3 ppm) and fiveteen methyl or methine carbons. However, no methylene group was present

Table 2. Assignment of ^{1}H and ^{13}C NMR spectra of **1** (500 MHz, in $CDCl_3$, chemical shifts in ppm, multiplicity in parentheses, coupling constants in Hz).

Position	1		
	δ_C	δ_H	COSY
1	18.6 (q)	1.77 s	-
2	142.2 (s)	-	-
3	26.1 (q)	1.72 s	-
4	120.7 (d)	4.82 d, 10.1	H-5
5	75.8 (d)	5.46 d, 10.1	H-4
6	60.5 (s)	-	-
7	202.9 (s)	-	-
8	53.1 (d)	3.23 d, br, 3.5	H-9, H-10
9	54.5 (d)	3.64 m	H-8, H-10, H-13
10	47.8 (d)	2.74 dd, 5.2, 7.8	H-9, H-11
11	71.5 (d)	4.99 dd, 5.2 br	H-10, H-12
12	40.4 (d)	3.55 m	H-11, H-13
13	72.7 (d)	3.80 dd, 9.5, 1.5; 2.54 br (OH)	H-12, H-9
14	61.0 (d)	3.51 dd, 2.9, 0.5	H-12, H-15
15	53.2 (d)	3.29 d, 3.2	H-14
16	192.8 (s)	-	-
17	132.5 (s)	-	-
18	139.6 (d)	6.73 dd 5.3, 2.4 (allyl)	H-19
19	40.9 (d)	3.59 d, 5.3	H-18
20	77.3 (s)	-	-
21	26.6 (q)	1.26 s	-
22	24.7 (q)	1.15 s	-
23	49.1 (q)	3.02 s	-

Abbreviations: s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet, br: broad.

suggesting the oligocyclic structure of **1**. The chemical down-field shift of seven of the carbon signals (C-5, C-11, C-13, C-14, C-15, C-20, C-23, see Table 2) proposed their binding to oxygen. The unusual upfield shifts of the oxygen-bonded C-14 (61.0 ppm) and C-15 (53.2 ppm) were explained readily by the occurrence of an epoxide and the neighbourhood of a keto group (C-16). For structural assignment the C,H long-range coupled NMR spectrum (HMBC) was of pivotal importance (Fig. 2). The presence of an endoperoxide in **1** was suggested by the upfield shift of protons H-5 (5.50 ppm) and H-11 (5.01 ppm) indicating the presence of a cyclic system and the occurrence of a broad hydroxyl proton signal at 2.56 ppm which was assignable doubtlessly to C-13, due to the observable NOE couplings with neighboured protons. Ring closure between C-17 and C-8 was suggested by the quartet structure of H-18 with $^{3}J_{H\text{-}18,H\text{-}19}=5.3$ Hz and allylic $^{4}J_{H\text{-}8,H\text{-}18}=1.4$ Hz.

The relative stereochemistry of **1** as shown in Fig. 1 was settled on the basis of the observable $^{1}H,^{1}H$-COSY and

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

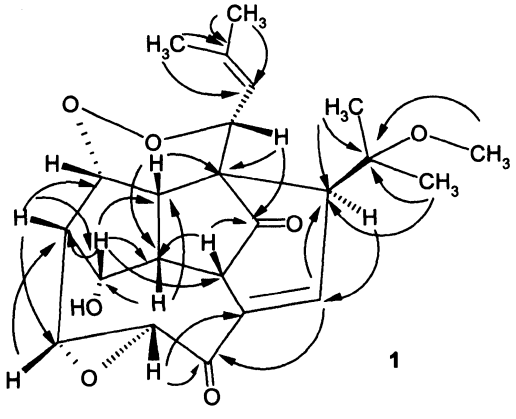
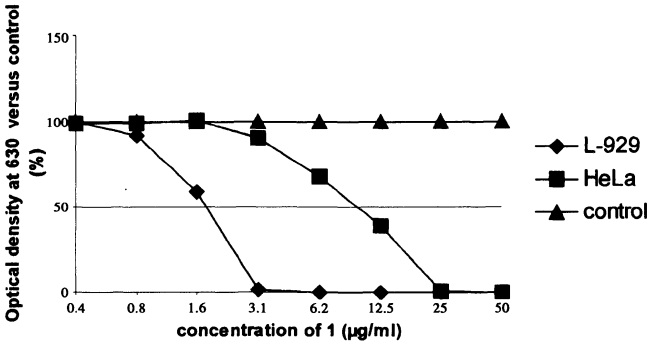


Fig. 3. Antiproliferative effect of hexacyclinol (**1**) on L-929 cells and cytotoxic effect on Hela cells.



NOESY correlations. Strong transannular NOE signals were observed between the C-13 hydroxyl proton and H-14/H-15 suggesting relative α-position for the former and the epoxide (Fig. 1b). Otherwise the visible NOE's between H-11/H-13, H-13/H-10, H-12/H-9 and H-8/H-10 supported a relative β-stereochemistry of protons at these positions. Strong NOE correlations were observed between H-5 and H-10 but not between H-5 and H-19 proposing relative β-configuration the side chain at C-19.

Hexacyclinol (**1**) thus appears as an unusual oligocyclic structure containing epoxide and endoperoxide groups, in addition to double bonds and rings. It possesses a spherical shape as was shown by 3D optimization (Fig. 1b). Coproduction of terpenoid metabolites by *Panus rudis* and the presence of dimethyl substituted carbons (C-2, C-20) indicated that biosynthesis of **1** occurred *via* the terpenoid pathway. **1** displayed moderate antibiotic activity against Gram-positive bacteria such as *Staphylococcus aureus* SG511 and *Bacillus subtilis* ATCC6633 with MIC 15.6 μg/ml. However, hexacyclinol (**1**) displayed strong biological activities as inhibitor of oxidant generation in zymosan-stimulated polymorphonuclear neutrophil leukocytes (PMNL)[5]. Thus potent inhibition of respiratory burst activity in PMNL was measured in concentrations 4 μg/ml to 40 μg/ml.

The growth of L-929 and K 562 cells was inhibited by 50% in concentration of 1.4 and 0.4 μg/ml, respectively. The flat shape of dose-response curve suggested an antiproliferative effect of **1** (Fig. 3). Hexacyclinol (**1**) was moderately cytotoxic to HeLa cells (CC_{50}=10 μg/ml). Experimental determination of antiproliferative and cytotoxic effects was carried out as described earlier[6]. Moreover **1** displayed moderate inhibitory activity against *Plasmodium falciparum* with IC_{50}=2.49 μg/ml. The IC_{50} was determined after 72 hours using cultures of 2.5% haematocrit and 0.3% initial parasitaemia[7].

It can be suggested that **1** owes its biological activities to the occurrence of reactive groups such as epoxide and endoperoxide. Together with artemisinin, ascaridiol and ergosteryl peroxide the hexacyclinol (**1**) represents one more of the few examples of naturally occurring endoperoxides[3,4].

Acknowledgements

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