ANTIBIOTICS FROM BASIDIOMYCETES. 26†

PHLEBIAKAURANOL ALDEHYDE AN ANTIFUNGAL AND CYTOTOXIC METABOLITE FROM *PUNCTULARIA ATROPURPURASCENS*

H. ANKE

University of Kaiserslautern, Department of Biotechnology,
D-6750 Kaiserslautern, FRG

I. CASSER and W. STEGLICH

University of Bonn, Department of Chemistry,
D-5300 Bonn, FRG

E.-H. POMMER

BASF AG,
D-6703 Limburgerhof, FRG

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Phlebiakauranol aldehyde and the corresponding alcohol were isolated from cultures of *Punctularia atropurpurascens*. The aldehyde but not the alcohol exhibited strong antifungal activity against several phytopathogens as well as antibacterial and cytotoxic activities. Two acetylated derivatives prepared from the aldehyde showed only very weak antifungal and antibacterial and moderate cytotoxic activities. We therefore assume, that the aldehyde group together with the high number of hydroxyl groups are responsible for the biological activity of the compound.

During our screening of Basidiomycetes for the production of compounds with antifungal activity, two metabolites, phlebiakauranol aldehyde (**1**) and the corresponding alcohol (**2**), were isolated from cultures of the conidial state of a Basidiomycete, strain HA 193-82. Phlebiakauranol aldehyde had been previously described as derived by heating from phlebiakauranol (**3**), a metabolite of *Phlebia strigosozonata*[2], **2** is a new kaurane derivative.

Due to limited availability little is known on the biological activity of **1** and **3**. A weak inhibition of *Staphylococcus aureus* had been reported for **3**[2]. In the following the producing organism and the biological activities of **1** and **2** and two derivatives prepared from **1** are described.

Materials and Methods

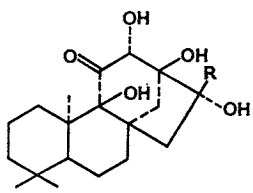
Culture Media

All fungi were grown and kept on YMG-medium[3]. For the production of **1** BAF-medium[4] was used. Bacteria were grown on nutrient broth (NB). For the evaluation of the antimicrobial activity *Streptomyces viridochromogenes* and fungi were grown on YMG or on minimal medium MM3[5], the other bacteria on NB and MM2[3].

Assays

Activity against phytopathogenic fungi was tested in the glasshouse. The test plants were grown in compost soil in 8 cm diameter pots. The compounds were formulated as solutions in acetone, with

† See ref 1.



1 R = CHO
2 R = CH_2OH
3 R = $CHOCH_3$ (OH)
4 R = CHO
Diacetate (position undetermined)
5 R = CHO
Triacetate (position undetermined)

the addition of a wetting agent. The solutions were diluted to the required concentrations immediately before use. The formulations were sprayed onto the leaves. The sprays were applied to maximum retention. For most of the tests the compounds were applied to the leaves 1 day before the plants were inoculated with the disease organism. An exception was the test with *Plasmopara viticola* in which the plants were inoculated 7 days after treatment. The plant pathogens were applied by spraying spore suspensions onto the leaves of the test plants. After inoculation, the plants were placed in an appropriate environment to allow the infection to proceed and then incubated until the disease symptoms were ready for assessment. The period between inoculation and assessment varied from 5 to 7 days, depending on the development of the disease and the environment. The disease control was recorded as percentage of leaf surface infected.

Tests for hemolytic activity were carried out as previously described[6]. Incorporation of radioactive precursors into DNA, RNA, and protein of bacteria, yeasts and Ehrlich carcinoma ascitic (ECA) cells were performed as described before[7].

Preparation of the Acetylated Derivatives from **1**

To a solution of **1** (0.8 g) in Ac_2O (10 ml) 5 mg 4-(dimethylamino)pyridine and triethylamine (3 ml) were added. The mixture was stirred for 3 hours under nitrogen atmosphere. The mixture was poured into ice water and extracted with EtOAc. The organic solution was washed with water, dried with Na_2SO_4 and concentrated. Flash chromatography of the residue with petroleum ether (40~60) - EtOAc (1 : 1) on silica gel yielded a white powder of phlebiakauranol triacetate **5** (46%): $[\alpha]_D^{22}$ +71° (*c* 0.8, $CHCl_3$); mp 115~118°C; IR (KBr) cm^{-1} 3500 (w), 2945 (m), 1765 (sst), 1740 (sst), 1460 (w), 1370 (st), 1230 (sst), 1205 (sst), 1070 (m), 890 (w); ^1H NMR (400 MHz, $CDCl_3$) δ 0.81 (3H, s, 18-CH_3), 0.89 (3H, s, 19-CH_3), 1.27 (3H, s, 20-CH_3), 2.02 (3H, s, $COCH_3$), 2.08 (3H, s, $COCH_3$), 2.14 (3H, s, $COCH_3$), 1.10~2.30 (13H), 3.20 (1H, dd, J=13.0 and 2.5 Hz, 14-H), 3.30 (1H, dd, J=16.5 and 2.0 Hz, 15-H), 5.52 (1H, d, J=2.5 Hz, 12-H), 9.32 (1H, s, 17-CHO); MS (AEI-MS50, 180°C) *m/z* 492.2386 (M^+, 0.70%, calcd $C_{26}H_{36}O_9$ 492.2359), 450 (0.99, $C_{24}H_{34}O_8$), 408 (0.32, $C_{22}H_{32}O_7$). Phlebiakauranol diacetate (**4**) was eluted as a minor by - product (18%, yellowish oil): MS (180°C) *m/z* 450.2243 (M^+, 0.04%, calcd $C_{24}H_{34}O_8$ 450.2253). The position of the acetyl groups in **4** and **5** is undetermined.

Results

Producing Organisms and Production of **1** and **2**

Strain HA 193-82 was isolated from a *Ganoderma* species collected in South Africa. An identical culture was obtained from a soil sample collected in the same area.

On agar slants strain HA 193-82 grows with clamp connections and produces conidia of the *Ptychogaster* type[8]. This anamorph might belong to one of the following teleomorphs: *Oligosporus*, *Tyromyces* or *Punctularia*[9]. Several strains from our culture collection of Basidiomycetes belonging to the genus *Tyromyces* were screened for the production of kaurane derivatives, but none was found to be positive. Comparison with four strains of the genus *Punctularia* from CBS revealed that our strain is similar to *Punctularia atropurpurascens* CBS 407.70. Both strains produce dark red conidia on agar cultures. In submerged cultures the production of conidia is dependent on the carbon-

nitrogen ratio of the medium; high content of amino acids favors the production of conidia and pigments of the terphenylquinone type[10]. The production of **1** is best in media with a high carbohydrate content. The production of kauranes and pigments by different strains of the genus *Punctularia* is depicted in Table 1.

For the production of **1**, strains HA 193-82 or CBS 407.70 were grown on BAF-medium at 22°C. The conidia from two agar plates (9 cm) were used to inoculate a 20-liter fermentor (Biolafitte, Paris) which was aerated with 2 liters/minute at 200 rpm. The antibiotic production was monitored by pipeting 15 μl of culture filtrate on 6 mm paper discs, which were applied to agar plates seeded with *Acinetobacter calcoaceticus* or *Bacillus brevis*. The production of **1** started after 4~6 days of growth and was accompanied by a heavy foaming of the culture. During the fermentation the pH of the culture broth dropped from 5.5 to 3.5. When the carbon sources were used up, the pH increased and the culture had to be harvested due to extremly heavy foaming. A typical fermentation diagram is depicted in Fig. 1. The mycelia were separated on a Büchner funnel. The culture broth was extracted with ethyl acetate; the organic solvent was evaporated under reduced pressure. The solid red residue was washed with petroleum benzine and the crude product was crystallized from chloroform, yielding **1** as a white product, which was identified by its UV, IR and NMR spectra.

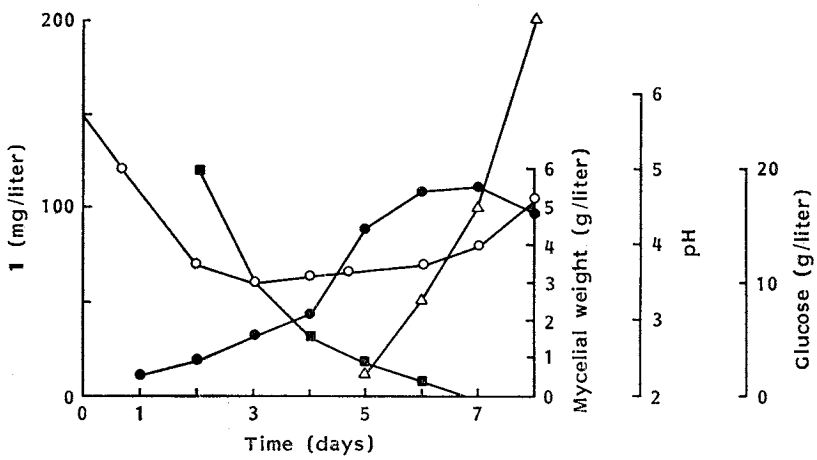
1 is easily soluble in MeOH, after cooling of the solution large crystals of **3** can be obtained. On TLC plates **1** can be visualized by spraying with vanillin - sulfuric acid (purple). Mycelial extracts,

Table 1. Production of **1** and terphenylquinones in submerged cultures of different strains and species of the genus *Punctularia* in BAF-medium.

Organism	**1**	Phlebia-rubrone	Hydroxy-phlebia-rubrone	Dihydroxy-phlebia-rubrone	Trihydroxy-phlebia-rubrone
Ptychogaster rubescens CBS 407.70	++	++	−	+	+
HA 193-82	+++	++	+	+	+
Punctularia atropurpurascens CBS 407.70	+++	++	+	+	+
P. strigosozonata CBS 345.34	+	++	−	−	−
P. strigosozonata CBS 308.78	+	+	−	+	+

−: <1 mg/liter, +: 1~10 mg/liter, ++: 10~50 mg/liter, +++: >50 mg/liter.

Fig. 1. Fermentation diagram of strain HA 193-82 in BAF-medium.
△ **1**, ● mycelial weight, ■ glucose, ○ pH.



obtained by extraction with acetone, contained **2** as a minor byproduct, easily recognized by the same color reaction with vanillin - sulfuric acid. On one occasion the mycelia from a 20-liter fermentor however contained only this compound. After chromatography on silica gel with CH_2Cl_2 and CH_2Cl_2 - MeOH (9 : 1) as eluent, **2** was obtained as white powder (30 mg/20 liters).

Identification of **1** and **2**

Physico-chemical Properties

Phlebiakauranol Aldehyde (**1**): Colorless solid; mp 198~200°C; $[\alpha]_D^{22}$ +120° (*c* 1.1, DMSO); IR (KBr) cm^{-1} 3440 (sst), 2930 (sst), 1705 (st), 1455 (w), 1440 (w), 1150 (m), 1095 (st), 1045 (st); ^1H NMR (400 MHz, acetone-d_6 - D_2O) δ 0.75 (3H, s, 18-CH_3), 0.79 (3H, s, 19-CH_3), 1.20 (1H, d, J= 15.5 Hz, 15-H), 1.35 (3H, s, 20-CH_3), 0.90~1.80 (10H), 1.86 (1H, dd, J=12.0 and 2.5 Hz, 14-H), 2.73 (1H, dd, J=15.5 and 2.0 Hz, 15'-H), 2.85 (1H, dm, J=12.0 Hz, 5-H), 2.94 (1H, dd, J=12.0 and 2.0 Hz, 14'-H), 3.62 (1H, d, J=2.5 Hz, 12-H), 9.47 (1H, s, 17-CHO); MS (AEI-MS 50, 180°C) *m/z* 366.2065 (M^+, 13.23%, calcd $C_{20}H_{30}O_6$ 366.2043), 348 (19.49, $C_{20}H_{28}O_5$), 276 (55.45, $C_{17}H_{24}O_3$).

Phlebiakauranol Alcohol (**2**): Colorless solid; mp 206°C (dec); $[\alpha]_D^{22}$ +96° (*c* 1.2, DMSO); IR (KBr) cm^{-1} 3420 (sst), 2940 (sst), 1700 (st), 1460 (m), 1390 (m), 1170 (st), 1100 (st), 1035 (sst); ^1H NMR (acetone-d_6 - D_2O) δ 0.75 (3H, s, 18-CH_3), 0.78 (3H, s, 19-CH_3), 1.21 (1H, d, J=16.0 Hz, 15-H), 1.38 (3H, s, 20-CH_3), 1.78 (1H, dd, J=12.0 and 2.5 Hz, 15'-H), 2.86 (1H, dd, J=12.0 and 1.5 Hz, 14'-

Table 2. Antibacterial and antifungal activity of **1**, **2**, **4** and **5** in the serial dilution assay. Size of inoculum: 5×10^5 cells of spores per ml.

Organism	Medium*	Minimal inhibitory concentrations (μg/ml)			
		1	**2**	**4**	**5**
Bacteria					
Acinetobacter calcoaceticus DSM 30006	I	5~10	>50	50	>50
Bacillus brevis ATCC 9999	I	5~10	>50	50	>50
B. subtilis ATCC 6633	I	10	>50	50	>50
B. subtilis ATCC 6051	I	50	>50	>50	>50
B. subtilis ATCC 6051	II	2	>50	20	>50
Escherichia coli K-12	I	>50	>50	>50	>50
E. coli K-12	II	30	>50	>50	>50
Micrococcus luteus	I	50	>50	>50	>50
Proteus vulgaris	I	20	>50	>50	>50
P. vulgaris	II	2	>50	20	50
Streptomyces viridochromogenes ETH 23575	I	10	>50	50	>50
Yeasts and fungi					
Botrytis cinerea	III	50	>50	>50	>50
Fusarium oxysporum	IV	>50	>50	>50	>50
Mucor miehei	IV	>50	>50	>50	>50
Nematospora coryli	III	2	>50	20	50
N. coryli	IV	<1	50	5	20
Paecilomyces varioti	III	>50	>50	>50	>50
P. varioti	IV	5	>50	50	>50
Penicillium notatum	IV	>50	>50	>50	>50
Saccharomyces cerevisiae	IV	>50	>50	>50	>50
S. cerevisiae is1	III	30	>50	>50	>50
S. cerevisiae is1	IV	1	>50	50	>50

* Medium I: Nutrient broth, II: MM2, III: YMG, IV: MM3.

H), 3.21 and 3.30 (2H, AB-system, J=12.0 Hz, 17-CH_2OH), 3.97 (1H, d, J=2.5 Hz, 12-H); MS (170°C) m/z 368.2185 (M^+, 22.04%, calcd $C_{20}H_{32}O_6$ 368.2198), 350 (9.49, $C_{20}H_{30}O_5$), 276 (100, $C_{17}H_{24}O_3$).

Biological Properties

As depicted in Tables 2 and 3, **1** showed broad antibacterial and a more selective antifungal activity, whereas **2** was devoid of inhibitory activity. The acetylated derivatives **4** and **5** were active only at high concentrations and only when the test organisms were grown on minimal media (MM). In complex media **4** and **5** showed no antifungal activity at all. The antimicrobial activities of **1** were also more pronounced when the organisms were grown on MM. The MIC against *Bacillus subtilis* decreased from 50 μg/ml on NB to 2 μg/ml on MM2. **1** exhibited good activity against phytopathogens under laboratory conditions (Table 3) as well as in greenhouse experiments. In the greenhouse trials very good results against *Phytophthora infestans* on tomatoes and *Plasmopara viticola* on grapevine

Table 3. Antifungal activity of **1**, **2**, and the acetylated derivatives **4** and **5**.
Plate diffusion assay. 10 or 100 μg were applied onto filter disks. The disks were placed on plates seeded with the test fungus in the top layer of the agar.

Organism	Medium*	Diameter of inhibition zone (mm)				
		1		**2**	**4**	**5**
		10 μg	100 μg	100 μg	100 μg	100 μg
Absidia glauca (+)	I					
	II		15			
A. glauca (−)	I					
	II		14			
Ascochyta pisi	I					
	II		20			
Alternaria porri	I		10			
	II	7	20		17	15
Botrytis cinerea	I		8			
Curvularia lunata	I		10			
	II	15	30		20	17
Eurotium cristatum	I					
	II	7	17		12	10
Fusarium fujikuroi	I		10			
	II		14		13	9
F. oxysporum	I		10			
	II		15		13	10
Nematospora coryli	I	10	>30		7	
	II	30			20	15
Neurospora crassa arg, aga	I	7	20			
Paecilomyces varioti	I					
	II	9	20		10	10
Phoma clematidina	I	15	30			
	II	20	>30		16	14
Phytophthora infestans	I		10			
	II	9	20			
Saccharomyces cerevisiae isl	I	7	21		8	
Ustilago nuda	I					
	II	8	25			
Venturia inaequalis	I					
	II	8	18		8	

* I: YMG-medium, II: synthetic medium.

were obtained. **1** proved to be as effective as Mitiram, a commercial fungicide (Table 4). Good results were also observed with *Septoria nodorum* on wheat and *Piricularia oryzae* on rice plants, whereas *Botrytis cinerea* on pepper, *Alternaria solani* on tomatoes, and *Erysiphe graminis* and *Puccinia recondita* on wheat were not affected.

1 as well as the acetylated derivatives showed cytotoxic effects. In ECA cells growth was reduced and lysis of the cells was observed. **2** had no effect. In ECA cells DNA, RNA and protein syntheses were completely inhibited at 18 μg/ml of **1**, 50% inhibition was observed at 8~10 μg/ml (Table 5). A similar inhibition of DNA, RNA and protein syntheses was obtained with exponentially growing cells of *B. brevis*, the concentrations needed for 50% inhibition were 38~48 μg/ml.

Table 4. Effects of phlebiakauranol aldehyde on late blight (*Phytophthora infestans*) of tomatoes and downy mildew (*Plasmopara viticola*) of grapevine (greenhouse trials).

Treatments	Rate mg a.i./liter	% Disease control (inoculation 7 days after treatment)	
		Phytophthora infestans	*Plasmopara viticola*
Phlebiakauranol aldehyde	1,000	100	90
	500	90	85
	250	80	75
	125	65	70
Metiram	1,000	95	95
	500	90	95
	250	75	80
	125	70	80
Untreated	—	70% of leaf surface infected	80% of leaf surface infected

a.i.: Active ingredient.

Table 5. Inhibitory activity of **1**, **2**, **4**, **5** and effects on macromolecular syntheses. Concentrations needed for 50% inhibition (μg/ml).

	1	**2**	**4**	**5**
Ehrlich carcinoma cells				
Growth inhibition IC_{50}	2	>30	5~10	5~10
DNA-synthesis	9	>30	22	15
RNA-synthesis	8	>30	22	15
Protein-synthesis	10	>30	22	14
Bacillus brevis				
MIC	5~10	>50	50	>50
DNA-synthesis	48	>50	>50	>50
RNA-synthesis	38	>50	>50	>50
Protein-synthesis	42	>50	>50	>50
Nematospora coryli				
MIC	2	>50	>50	>50
DNA-synthesis*				
RNA-synthesis	12	NT	NT	NT
Protein-synthesis	18	NT	NT	NT

* No incorporation due to lack of thymidine kinase.
NT: Not tested.

Discussion

In the present study **1** has been isolated together with **2** from cultures of *P. atropurpurascens*. *Ptychogaster rubescens* and *P. strigosozonata* were also found to produce **1** but not **2**. In the presence of methanol **1** is easily converted to **3** previously isolated from *Phlebia strigosozonata*[2]. *Phlebia strigosozonata* has been transferred to *Phaeophlebia strigosozonata* (=*Punctularia strigosozonata*)[11]. Our results clearly show, that **1** is the original metabolite not only from *P. atropurpurascens* but also of *P. strigosozonata* and the anamorph *Ptychogaster*; **3** is an artifact derived from **1**, as already suspected by LISY *et al.*[2].

For the antimicrobial activity the aldehyde group together with a high number of hydroxyl groups are essential. The alcohol **2** is inactive and the acetylated derivatives **4** and **5** showed only very weak activity. The cytotoxic activity of **4** and **5** however was only slightly reduced. The lytic activity observed in cell cultures can be deduced from the structure of **1**: It contains a highly oxygenated hydrophilic part and a hydrophobic part.

Kauranes are not very common among the secondary metabolites of mushrooms[12,13]. Up to now strains of the genus *Punctularia* and their anamorphs are the only producers.

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