Bioactive Sesquiterpenes from the Basidiomycete *Resupinatus leightonii*

Frank Eilbert,[†] Michaela Engler-Lohr,[†] Heidrun Anke,*,[†] and Olov Sterner[‡]

Institute for Biotechnology and Drug Research (IBWF e.V.), D-67663 Kaiserslautern, Germany, and Department of Organic Chemistry 2, Lund University, P.O. Box 124, S-22100 Lund, Sweden

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1(10),4-Germacradiene-2,6,12-triol (**1**), a new germacrane sesquiterpene, and 1,6-farnesadiene-3,10,11-triol (**2**), a known nerolidol derivative, were isolated from submerged cultures of the basidiomycete *Resupinatus leightonii*. Both compounds inhibited cAMP-induced appressorium formation in *Magnaporthe grisea* and showed cytotoxic activity. The structure of **1** was determined by NMR and mass spectroscopic methods.

The formation of melanized appressoria is a prerequisite for the invasion of host plants by the plant pathogenic fungus *Magnaporthe grisea* (Herbert) Barr (anamorph *Pyricularia oryzae* Cavara). Appressorium formation can be induced by activators of different signal transduction pathways such as cAMP[1] or 1,2-dioctanoyl-*sn*-glycerol[2] (DOG). Inhibitors not only might be valuable tools for investigating mechanisms and pathways leading to infection structure formation but could also lead to new targets of novel fungicides and therefore may be of interest as plant protectants. In the course of a screening for inhibitors of appressorium formation in germinating conidia of *M. grisea*, two compounds were isolated from fermentations of the basidiomycete *Resupinatus leightonii* as inhibitors of a cAMP-induced appressorium formation. Metabolites were identified as 1(10),4-germacradiene-2,6,12-triol (**1**) and 1,6-farnesadiene-3,10,11-triol (**2**). In this paper the isolation and structure determination of **1** is reported and the biological activities of **1** and **2** are described.

1

2
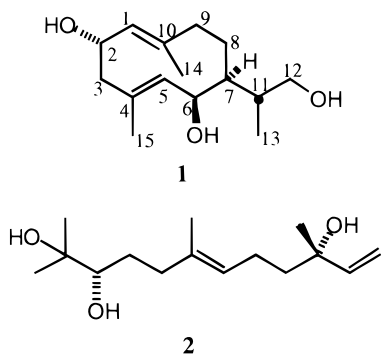
The two metabolites were isolated from a crude extract of culture fluids from submerged cultures of the basidiomycete *Resupinatus leightonii* P.D. Orton (Tricholomataceae) by bioactivity-guided fractionation according to details given in the Experimental Section. *R. leightonii* has previously been reported to produce the 14-noreudesmanes panellon and panellol.[3] Spectral analysis of the newly isolated compounds revealed that the farnesane **2** is a known metabolite, previously reported from *Mikania microptera*[4] and reported here for the first time from a fungus. The NMR data determined for **2** in this investigation are
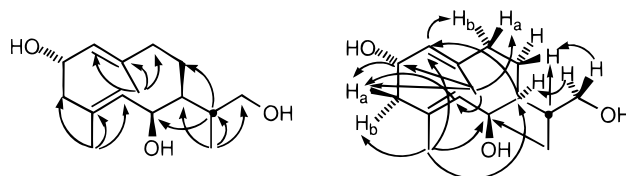

Figure 1. Pertinent HMBC (left) and NOESY (right) correlations observed with compound **1**.

identical to those reported in the same solvent and at the same frequency.[4] The second active metabolite was found to be a new germacrane sesquiterpene **1**, whose structure was determined by spectroscopic methods. High-resolution MS data suggested an elemental composition of $C_{15}H_{26}O_3$, which was confirmed by 1D NMR data. The germacrane skeleton, the positions of substituents, and unsaturations were determined by COSY and HMBC correlations (pertinent HMBC correlations are shown in Figure 1). Especially important are the long-range correlations between H_3-14 and C-1, C-9, and C-10, between H_3-15 and C-3, C-4, and C-5, and between H-11 and C-6, C-7, and C-8. The latter established the connection between C-6 and C-7, which was not evident from their small $^1H-^1H$ coupling constant. The relative stereochemistry was suggested by correlations observed in the NOESY spectrum (Figure 1). Both double bonds are *E*, as H-1 gives a NOESY correlation to Hb-9, not to H_3-14, and H-5 correlates to Ha-3, not to H_3-15. H_3-14 give NOESY correlations to H-2, Ha-3, H-5, and Ha-9, indicating that all these protons are located on the same side of the molecule in the most stable conformation. The C-4 methyl group would then be on the opposite side together with H-1, Hb-3, and H-6, all which give NOESY correlations to H_3-15. Also H-7 should be located on this side, as it gives NOESY correlations with H-1 as well as H_3-15, indicating H-7 can be regarded as axial while the hydroxylated isopropyl group is equatorial. Inspection of a Dreiding model for the proposed structure reveals a dihedral angle between H-6 and H-7 is close to 90°, which fulfills the NOESY correlations discussed above, and agrees with the magnitude of $^1H-^1H$ coupling between these two protons. Finally, H_3-13 correlates strongly with H-6, while H-11 and Ha-12 correlate with Hb-8, and Hb-12 correlates with H-7, in support of the suggested stereostructure.

When germinating conidia of *M. grisea* were tested, appressorium formation induced by the hydrophobic surface of GelBond sheets or induced by plant wax components such as 1,16-hexadecanediol on a hydrophilic (noninductive) surface was not inhibited by compound **1** or **2**. Only when the cAMP analogue chlorophenylthio-cAMP was used

* To whom correspondence should be addressed. Tel.: +49-631 31672 10. Fax: +49-631 31672 15. E-mail: anke@ibwf.uni-kl.de.
† IBWF e.V.
‡ Lund University.

Table 1. Cytotoxicity of **1** and **2** Isolated from *R. leightonii* (IC_{50} Values in μg/mL)

compound	cell line			
	Jurkat	HL60	HeLa S3	B16-F1
1	20	10	>100	>100
2	20	5	75	75

as inductor on a hydrophilic surface was the formation of infectious structures prevented by either compound. For 50% inhibition, 50 μg/mL of **1** or 10 μg/mL of **2** was needed. This is the first report of natural compounds interfering with cAMP-mediated signal transduction leading to appressorium formation in *M. grisea*. Antifungal or antibacterial activities were not detected, but moderate cytotoxic activities are listed in Table 1.

Experimental Section

General Experimental Procedures. Preparative HPLC was carried out on a JASCO HPLC (PU 980, MD 910); analytical HPLC was carried out on a Hewlett-Packard 1090 Type II with LiChrosphere RP18 (10 μm; 125 × 4 mm) and a H_2O−MeOH gradient. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX500 spectrometer with an inverse multinuclear 5-mm probehead equipped with a shielded gradient coil. Spectra were recorded in $CDCl_3$, and solvent signals [δ_H 7.26 and δ_C 77.0 ppm] were used as reference. COSY, HMQC, and HMBC experiments were recorded with gradient enhancements using sine-shaped gradient pulses. For 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{CH} =$ 145 Hz and $^nJ_{CH} =$ 10 Hz. Raw data were transformed, and spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). MS were recorded with a JEOL SX102 spectrometer, while UV and IR spectra were recorded with a Perkin-Elmer λ 16 and a Bruker IFS 48 spectrometer. Melting points (uncorrected) were determined with a Reichert microscope, and optical rotation was measured with a Perkin-Elmer 141 polarimeter at 22 °C.

Producing Strain. Fruiting bodies of *R. leightonii* were collected in Hinterstein, Germany. Strain TA88026 is deposited in the culture collection of LB Biotechnology, University of Kaiserslautern. For maintenance on agar slants the fungus was grown on a medium consisting of yeast extract 4 g/L, malt extract 10 g/L, glucose 4 g/L (YMG), pH 5.5.

Fermentation and Isolation. Fermentation was carried out in YMG medium at 24 °C in a 20-L fermentor (Braun Biostat U) with aeration (3.0 L air/min) and agitation (120 rpm); 200 mL of a well-grown culture (10 days) in the same medium was used as inoculum. After 13 days the culture was harvested. Mycelium containing none of the metabolites was separated from the culture fluid and discarded. Compounds were extracted from the culture fluid by adsorption onto HP 21 resin (Mitsubishi). After washing the resin with 2 L of H_2O, active compounds were eluted with 1.5 L of acetone. The crude extract (2.4 g) obtained by concentration was fractionated on silica gel (Merck 60, 63−200 μm) using cyclohexanes−EtOAc with increasing EtOAc concentrations. Fractions containing the two compounds were eluted with cyclohexanes−EtOAc, 1:1. Final purification was achieved by preparative HPLC (LiChrospher RP18, 7 μm, column size 250 × 25 mm, flow rate 5 mL/min) using a H_2O−MeOH gradient (0−70% MeOH in 80 min; retention time 35 min (**1**) and 51 min (**2**)). From 16 L of culture filtrate 43 mg of **1** and 9 mg of **2** were obtained.

1(10),4-Germacradiene-2,6,12-triol (1). colorless oil; $[\alpha]_D$ −11° (*c* 0.9 in $CHCl_3$); UV (MeOH), λ_{max} (ϵ) no maxima above 210 nm; IR (KBr) 3377, 2918, 1663, 1447, 1383, 1308, 1261, 1235, 1187, 1144, 1109, 1077, 1026, 1007, 926, 864, 842, 792, 668, and 549 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) 4.93, d, J_{5-6}=8.1, 5-H; 4.88, d, $J_{1-2} =$ 6.6, 1-H; 4.85, t, $J_{1-2} =$ 6.6, J_{2-3} = 6.4, 2-H; 4.68, d, $J_{5-6} =$ 8.1, 6-H; 3.75, dd, $J_{11-12a} =$ 2.6, $J_{12a-12b} =$ 11.1, 12-Ha; 3.56, dd, $J_{11-12b} =$ 6.0, $J_{12a-12b} =$ 11.1, 12-Hb; 2.42, dd, $J_{2-3} =$ 6.4, $J_{3a-3b} =$ 13.2, 3-Ha; 2.22, d, J_{3a-3b} = 13.2, 3-Hb; 2.13, m, 9-Ha; 1.77, m, 8-Ha; 1.77, m, 9-Hb; 1.71, m, 11-H; 1.61, s, 15-H_3; 1.52, s, 14-H_3; 1.28, m, 8-Hb; 1.16, m, 7-H; 1.06, d, $J_{11-13} =$ 7.1, 13-H_3; ^{13}C NMR (125 MHz, $CDCl_3$) 135.3 C-10; 132.4 C-5; 132.3 C-4; 128.5 C-1; 71.2 C-2; 67.1 C-6; 64.8 C-12; 50.5 C-7; 47.0 C-3; 40.9 C-9; 40.4 C-11; 25.9 C-8; 18.4 C-15; 17.6 C-14; 16.1 C-13. EIMS (70 eV), *m/z* (rel int.): 254.1888 (8%, M$^+$, $C_{15}H_{26}O_3$ requires 262.1882) 236 (13%), 221 (15%), 218 (16%), 192 (19%), 177 (27%), 159 (31%), 135 (28%), 109 (63%), 97 (100%).

Biological Assays. Inhibition of appressorium formation in germinating conidia of *M. grisea* strain P1 was measured as described previously.[5] Cytotoxic activities against Jurkat (ATCC TIB 152), HL60 (ATCC CCL 240), HeLa S3 (ATCC CCL 2.2), and B16-F1 (ATCC CRL 6323) were also assayed as described previously.[6]

References and Notes

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