Geniculol, a New Biologically Active Diterpene from the Endophytic Fungus *Geniculosporium* sp.[1]

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An endophytic fungus of the genus *Geniculosporium* was isolated from *Teucrium scorodonia*. The EtOAc extract of this isolate exhibited antialgal activities, as well as demonstrating an interesting profile during chemical screening. After mass cultivation of this fungus, in an optimized agar medium, two of the compounds responsible for the observed biological activity were isolated, the new diterpene geniculol (**1**), and the known fungal metabolite cytochalasin F (**2**). Geniculol is an unprecedented and irregular diterpenoid. For **1** and **2** complete ^{1}H and ^{13}C NMR data are reported. Both metabolites exhibit algacidal properties. Additionally, cytochalasin F was shown to be an inhibitor of photosynthesis, suggesting an ecological function in the plant−symbiont relationship for this secondary metabolite.

Endophytic fungi are to be found in all or almost all plants. These include trees, grasses, algae, mosses, and herbaceous plants.[2] Most fungal endophytes belong to the Ascomycetes and Fungi imperfecti. Under normal circumstances they live within the host plant without causing any noticeable symptoms of disease. When the host is stressed, however, some endophytes may become pathogenic. The delicate equilibrium between host and endophyte seems to be controlled in part by chemical factors, for example, herbicidal natural products produced by the fungi versus antifungal metabolites biosynthesized by the host plant.[3]

In 1993, Schulz et al.[2] established that the fungal endophytes of the plant *Teucrium scorodonia* belong to the genera *Achaetomium*, *Alternaria*, *Epicoccum*, *Fusarium*, *Nodulisporium*, *Sordaria*, and *Geniculosporium*. Recently it was shown that in a dual culture of *Geniculosporium* sp., (strain number 2121), with the callus of its host *T. scorodonia*, the fungus secreted low-molecular-weight compounds, able to diffuse into agar and finally causing tissue necrosis in the host plant.[4] The current study focused on the natural product investigation of the cultured endophyte *Geniculosporium* sp. (strain number 2121) and the assessment of the biological activity of the pure secondary metabolites. After a bioassay-guided fractionation scheme that monitored growth inhibition of *Chlorella fusca*, it was possible to isolate two compounds (**1** and **2**) that may be responsible for the observed activity.

Compound **1** was isolated as a clear oil, and analyzed for $C_{20}H_{26}O_6$ by mass spectrometry. Of the eight elements of unsaturation implied by the molecular formula of **1**, three were present in the form of multiple bonds, that is, one carbon−carbon double bond and two ester carbonyl groups, as discerned from the ^{13}C NMR and IR data [175.4 (s), 182.2 (s) ppm, 1760 cm^{-1}]; **1** is thus pentacyclic. Also evident from these data was the presence of two hydroxyl functions, one secondary [76.6 (d) ppm, 3420 cm^{-1}] and one tertiary [72.4 (s) ppm, 3420 cm^{-1}]. After association of all protons with their directly bonded carbon partners via a 2D-NMR shift correlated ^{1}H−^{13}C HMQC (a one bond correlation) measurement, it was possible from the HMBC spectrum to deduce two partial structures as indicated in fragment 1. From the ^{1}H−^{1}H COSY spectrum it was possible to develop fragment 2. The ester bond between the C-16 ester oxygen and C-15 was deduced from a detailed computer analysis of the HMBC spectrum. Connectivity between C-8 and C-9 followed by deduction and was confirmed by ^{1}H−^{1}H long-range couplings from H-16 to H-11 and from H_3-17 to H-11. The remaining points of closure to form one of the last two rings must be between one of the oxygens at C-6, C-9, or C-11 and C-19. From literature precedent[5−7] it was clear that the C-19 ester must bond to C-6, thus completing ring four. The two hydroxyl functions thus resided at C-9 and C-11. The fifth and final ring formed as a result of the closure between C-14 and C-15. NOE difference measurements yielded the relative stereochemistry at all chiral centers. Thus, irradiation of the resonance for H_3-17 caused enhancement of the resonances for H-11 and H-16, indicating all of them to be on the same side of the molecule. Irradiation of the resonance for H-16 caused enhancement of the resonance H-7. Further NOEs were observed between H_3-18 and H-5, and H-6, and between H-6, H-5, and H-7. These interactions indicated the γ- and δ-lactones as well as the 9-OH to be on the upper face of the molecule and the decalin ring to be trans-fused, as did the ^{1}H−^{1}H *W*-couplings between H-5 and H_3-20 and between H-5 and H-7. These deductions were further supported by ^{13}C NMR data comparisons made between the data for **1** and those for molecules with comparable structural entities.[5−7] For **1**, the trivial name geniculol is proposed.

After a complete analysis of its ^{1}H and ^{13}C NMR spectroscopic data, compound **2** was identified as cytochalasin F. Comparison of the current NMR data (see Table 1) with those published by Capasso et al.[8] indicated a significant revision of the published data to be necessary (see Table 1).

Growth inhibition of *C. fusca* in an agar diffusion bioassay[9] was employed to isolate two of the compounds (**1** and **2**) responsible for the antialgal activity. At a concentration of 50 μg, compounds **1** and **2** caused 2-mm and 1-mm radius inhibition zones, respectively, in this agar diffusion test. Compound **2** was further assessed for its ability to inhibit photosynthesis. The results of this experiment showed (see Table 2) that **2** significantly

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Table 1. ^{1}H (300 MHz, $CDCl_3$) and ^{13}C NMR (75.5 MHz, $CDCl_3$) Data[a] for **1** and **2**

carbon	^{1}H (**1**)	^{13}C (**1**)	^{1}H (**2**)	^{13}C (**2**)
1	1.26 (m), 1.82 (m)	26.7 (t)[b]		172.4 (s)
2	1.53 (m), 1.71 (m)	18.0 (t)		
3	1.52 (m), 2.15 (m)	28.2 (t)	3.68 (br ddd, J = 1.4, 6.3, 7.8 Hz)	53.9 (d)
4		42.4 (s)	2.86 (dd, J = 1.4, 5.6 Hz)	49.2 (d)
5	2.51 (d, J = 4.8 Hz)	43.8 (d)	2.34 (dq, J = 5.6, 7.3 Hz)	36.0 (d)
6	4.89 (dd, J = 4.6, 4.8 Hz)	72.1 (d)		57.4 (s)
7	6.37 (d, J = 4.6 Hz)	132.4 (d)	2.74 (d, J = 5.5 Hz)	61.0 (d)
8		137.1 (s)	3.13 (dd, J = 5.5. 9.9 Hz)	46.0 (d)
9		72.4 (s)		84.5 (s)
10		37.9 (s)	2.78 (d, J = 6.3 Hz)	45.1 (t)
			2.80 (d, J = 7.8 Hz)	
11	3.84 (s)	76.6 (d)	1.02 (d, J = 7.3 Hz)	12.8 (q)
12		42.4 (s)	1.21 (s)	19.6 (q)
13	1.33 (m),[c] 1.88 (m)[c]	25.2 (t)	6.09 (ddd, J = 1.6, 9.9, 15.2 Hz)	126.5 (d)
14	1.35 (m),[c] 1.74 (m)[c]	25.2 (t)	5.31 (ddd, J = 3.7, 10.9, 15.2 Hz)	135.9 (d)
15		175.4 (s)	1.72 (m)	41.8 (t)
			2.09 (dm, J = 13.5 Hz)	
16	4.76 (br s)	86.9 (d)	1.23 (m)	33.1 (d)
17	1.15 (s)	14.1 (q)	0.65 (m), 1.54 (m)	35.3 (t)
18	1.34 (s)	24.7 (q)	1.24 (m), 1.53 (m)	20.2 (t)
19		182.2 (s)	1.59 (m), 1.94 (ddd, J = 5.7, 7.2, 14.0 Hz)	34.8 (t)
20	0.93 (s)	22.0 (q)	4.55 (br s)	70.4 (d)
21			7.05 (dd, J = 3.8, 15.6 Hz)	152.5 (d)
22			5.93 (dd, J = 2.0, 15.6 Hz)	118.7 (d)
23				164.5 (s)
24			0.87 (d, J = 6.6 Hz)	20.4 (q)
O*H*	2.70 (br), 1.91 (br)	O*H* or N*H*	5.88 (br)	
1′				136.9 (s)
2′			7.15 (m)	129.3 (d)
3′			7.33 (m)	129.0 (d)
4′			7.26 (m)	127.2 (d)
5′			7.33 (m)	129.0 (d)
6′			7.15 (m)	129.3 (d)

[a] All assignments are based on extensive 1D and 2D NMR experiments, including COSY90, HMQC, and HMBC. [b] Multiplicity by DEPT, s = C, d = CH, t = CH_2, q = CH_3. [c] These resonances may be interchanged.

inhibited photosynthesis. This finding may thus account for the observed phytopathogenic activity of fungal metabolites in the original assay with the callus of its host plant *T. scorodonia*. Compound **1** decomposed prior to testing its influence on photosynthesis. Diterpenes of the pimarane class have been shown before, however, to be potent phytotoxins.[6]

Experimental Section

General Experimental Procedures. These procedures follow those previously published.[10]

Fungal Material. Leaves of *T. scorodonia* were collected from a site near Braunschweig, Germany. Twenty segments of each of 3−5 leaves were taken from different plants. To obtain the endophytes from within the leaves, surface sterilization with diluted formaldehyde (37−40% for 1 min) was used to kill the epiphytic fungi. The surface-sterilized leaf segments were incubated for 8 weeks at room termperature on a malt extract medium containing penicillin G and streptomycin sulfate. The mycelia that grew out of the surface of sterilized leaf segments were isolated and transferred to malt extract agar medium.

Mass Cultivation and Extraction of *Geniculosporium* sp. Conditions of culture for fermentation were chosen to enable an optimal production of active secondary metabolites. *Geniculosporium* sp. was cultured for 45 days in 30 × 1.8 L Fernbach flasks at room temperature, on biomalt (5% w/v) solid agar (0.3% w/v agar) medium. Each flask contained 250 mL of medium.

Extraction and Isolation. After cultivation, the resultant fungus and medium were homogenized with a Waring blender, diluted with H_2O (2 L), and then extracted three times with EtOAc (10 L). The combined organic extracts were evaporated in vacuo to dryness (4.7 g). The extract was separated by vacuum liquid chromatography (VLC) over Si gel applying gradient elution from hexane to EtOAc to MeOH to yield 10 fractions each of approximately 75 mL. These VLC fractions were screened monitoring growth inhibition of *C. fusca* and by ^{1}H NMR spectroscopy. VLC fraction 8 was found to be active and was further separated by HPLC (normal-phase silica with hexane−Me_2CO−CH_2Cl_2, 7:2:1, as eluent) to yield compounds **1** and **2**.

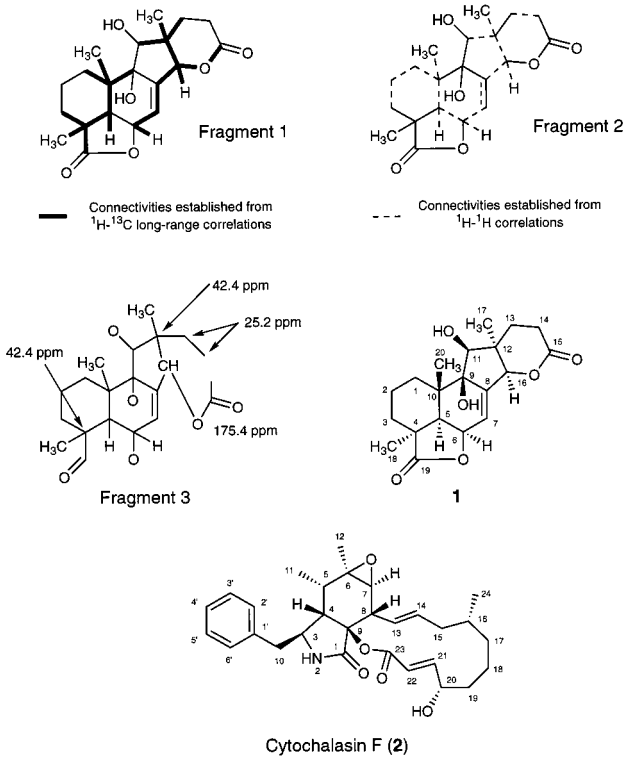

Table 2. Inhibition of the Photosynthetic Activity of the Green Alga *Chlorella fusca*

compound	photosynthesis μmol O_2/h × mL[a]		reduction of photosynthesis μmol O_2/h × mL (%)
	Before sample addition	After sample addition	
control: MeOH	0.2036	0.1572	0.0463 (22.8%)
Cytochalasin F (**2**) (0.2 mg/mL in MeOH)	0.4382	0.0257	0.4125 (94.1%)

[a] The rate of O_2 production was measured using an oxygen electrode (Clark-electrode).

Geniculol (1): isolated as a colorless oil (3.7 mg, 0.08% of extract); $[\alpha]^{20}_D$ −11.9° (*c* 0.37, $CHCl_3$); IR (film) ν_{max} 3420, 2925, 1760, 1205, 1045 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* $[M]^+$ 362 (<1), 287 (2), 285 (10), 243 (3), 277 (6), 109 (100); HRCIMS (isobutane) 362.1724 (calcd for $C_{20}H_{26}O_6$, 362.1729).

Cytochalasin F (2): isolated as a colorless oil (5.1 mg, 0.1% of extract); $[\alpha]^{20}_D$ −52.0° (*c* 0.51, $CHCl_3$) (lit.[11] −33.0°); ^1H and ^{13}C NMR data, see Table 1; remaining physical and spectroscopic data as previously published.[8,11−13]

Agar Diffusion Assays. These were performed as previously described.[9]

Inhibition of Photosynthesis. The green alga *C. fusca* was cultivated for 2 days in CP liquid medium (10 g glucose, 10 g yeast extract, 15 g agar, 1 L H_2O, pH adjusted to 6.2 prior to autoclaving); 50-mL portions of culture were incubated in 100-mL Erlenmeyer flasks on a shaker with 120 U/min at 20 °C. Before any measurements were made, the concentration of algae was adjusted to 1.8×10^7 cells/mL with a carbonate buffer (15% 0.1 mol/L Na_2CO_3, 85% 0.1 mol/L $NaHCO_3$). To achieve this, a known amount of algal culture was centrifuged for 5 min at 600 *g* and then suspended in the appropriate amount of buffer. The production of oxygen was measured with a P. J. Kipp and Zonen-Vertrieb-GmbH (Weisenau, Germany) model YSI 5331 oxygen electrode. The zero point of the electrode was established using Na_2SO_3 (50 g/L); 100% oxygen saturation was calibrated using distilled, air-saturated H_2O (250 mL of H_2O being aerated with compressed air for 1 h). The reaction chamber was then filled with 5 mL of the algal suspension in buffer and kept in the dark until the oxygen content declined to zero. Subsequently the suspension was illuminated, causing an increase of oxygen due to photosynthetic activity of the alga. At a concentration of 50% O_2 a 100-μL test solution was added. As control, the reaction of the alga toward the solvent MeOH was tested.

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Supporting Information Available: The IUPAC names and numbering of compounds **1** and **2** (1 page). Ordering information is given on any current masthead page.

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