Hydroxylation at Carbon-2 of *ent*-16-Oxo-17-norkauran-19-oic Acid by *Fusarium proliferatum*

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A new product of biotransformation of *ent*-16-oxo-17-norkauran-19-oic acid (**1**) by *Fusarium proliferatum* was isolated and identified as a 2β-hydroxy derivative (**2**). The structure of **2** was elucidated on the basis of spectroscopic data interpretation and single-crystal X-ray diffraction analysis. The allelopathic activity of compound **2** was evaluated on the growth of radicals and shoots of *Lactuca sativa* (lettuce). This is the first time that fungal hydroxylation at position C-2 has been reported on an *ent*-kaurane diterpene skeleton.

Gibberella fujikuroi is a fungal species that may involve a series of asexual lineages such as *Fusarium proliferatum*[1] and has been extensively used for the biotransformation of *ent*-kaurane diterpenes. One of the most interesting chemical modifications performed in *ent*-kaurane diterpenes by fungi is the hydroxylation of methylene-inactivated groups,[2] since it has been reported frequently that hydroxylated kaurane diterpenes are bioactive.[3] For example, a trihydroxylated *ent*-kaurane diterpene, isolated from the leaves of *Isodon wightii*, exhibited potent acetylcholinesterase and antioxidant activities.[4] Many different fungal species have shown to be useful for *ent*-kaurane hydroxylation such as *Rhizopus stolonifer*,[5,6] *R. arrhizus*,[7] and *Mucor plumbeus*,[8] but the use of *G. fujikuroi* is much more frequently accomplished.[9,10] Incubation of *ent*-kaurane derivatives of *G. fujikuroi* usually leads to the formation of the fungal secondary metabolite fujenal[11] as well as gibberellic acid[12] and other gibberellin-like compounds,[13] but many other hydroxylated *ent*-kaurane derivatives were produced using this fungal species. In addition, there have been derivatives reported that are hydroxylated at C-11, in either the α- or β-positions.[14] Positions C-15, C-16, C-18, and C-19[9] and C-7, C-13, and C-14[15] are frequently hydroxylated likewise. Some other positions of the *ent*-kaurane ring system may be hydroxylated rarely, such as position C-1,[16,17] but, to the best of our knowledge, no reports on the fungal hydroxylation of the *ent*-kaurane skeleton at position C-2 have been published so far.

In this work, we report the successful hydroxylation of *ent*-16-oxo-17-norkauran-19-oic acid (**1**) by *F. proliferatum* into a new C-2-hydroxylated derivative, determined as *ent*-2α-hydroxy-16-oxo-17-norkauran-19-oic acid (**2**) by one- and two-dimensional NMR spectroscopy (Figure 1). The proposed structure of **2** was confirmed by single-crystal diffraction analysis. The allelophatic activity of **2** was determined on the radicals and shoots of *Lactuca sativa*.

Incubation of *ent*-16-oxo-17-norkauran-19-oic acid (**1**) with *F. proliferatum* furnished a single compound (**2**) that crystallized as thin white needles, after column chromatography of the crude biotransformation extract. The structure of this compound was determined by detailed analysis of the 1D and 2D NMR (^{1}H, ^{13}C, DEPT, HMBC, HSQC, and NOESY) spectroscopic data (Table 1). Ketone and carboxyl carbonyls were not modified by the fungus since resonance signals at δ_C 221.3 and 180.4 were found in the ^{13}C NMR spectrum of compound **2**. The ^{1}H NMR spectrum of this substance showed the presence of eight methylene groups (one less than the starting material) and a signal of a methine carbon at δ 64.0, indicating a methylene group of the starting material to be hydroxylated. In the HSQC spectrum, this new methine signal showed a correlation to a multiplet centered at δ_H 4.81 (1H), corroborating the presence of a hydroxy group in the molecule of **2**. To establish the position and orientation of this new hydroxylated carbon, ^{13}C NMR signal assignments were carried out, with hydroxylation evident at C-2, since the resonance at δ_C 19.2,

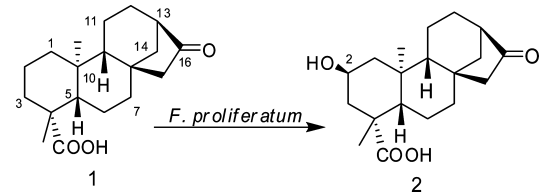

Figure 1. Biotransformation of *ent*-16-oxo-17-norkauran-19-oic acid (**1**) by *Fusarium proliferatum* into *ent*-2α-hydroxy-16-oxo-17-norkauran-19-oic acid (**2**).

Table 1. NMR Spectroscopic Data of **2**

position	δ_C, mult	δ_H (*J* in Hz)	HMBC	NOESY
1	50.9 CH_2	α: 2.52, dd (12.3, 2.7); β: 1.10, s	3; 20	2, 5, 9, 11 α, 20
2	64.0 CH	4.81, m	1; 3; 20	1α, 3 α, 18, 20
3	48.7 CH_2	α: 3.07, dd (12.3, 2.7); β: 1.39, s	18	2, 18
4	45.5 C		3; 20; 18	
5	56.7 CH	1.13, s	1; 3; 18	9, 18
6	21.6 CH_2	α: 2.04, m; β: 2.22, m	5; 9	6β, 20
7	41.6 CH_2	α: 1.71, m; β: 1.42, m	11; 15; 9	
8	42.8 C		7; 15; 9	
9	56.7 CH	1.17	11; 14; 15; 9	11β, 15α
10	41.4 C		20	
11	19.4 CH_2	α: 1.68, m; β:1.57, m	12; 20	1α, 9
12	29.9 CH_2	α:1.59, m; β: 1.93, m	11; 14	12α,14β
13	48.3 CH	2.31, m	11	11β, 14 β
14	37.7 CH_2	α: 2.10, m; β: 1.27, dd (12.0, 3.8)	13; 15; 9	14α, 20 14β, 15 β 14 β, 13
15	55.3 CH_2	α: 1.96, m; β: 2.00, d (3.8)	14	15β, 14β
16	221.3 C		15	
18	29.7 CH_3	1.44, s	3; 4; 9	
19	180.4 C		18	
20	17.9 CH_3	1.19, s	1; 5	

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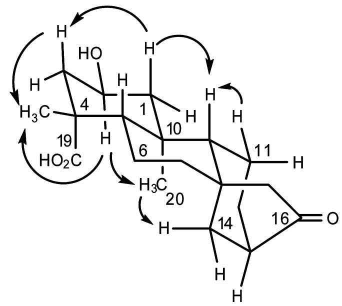

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Figure 2. Some correlations observed in the NOESY spectrum of compound **2**.

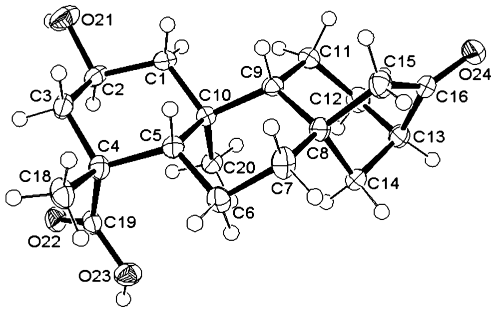

Figure 3. Perspective ORTEP drawing of compound **2**. Displacement ellipsoids are drawn at the 50% probability level.

corresponding to the C-2 signal in the starting material, was not present in the ^{13}C NMR spectrum of the product. The NOESY experiment (Figure 2) was used to determine the relative configuration of C-2, and H-2 (δ_H 4.81) correlated with H-20 (δ_H 1.19), a methyl group below the ring A plane, suggesting an α-orientation of H-2 and, therefore, a β-orientation of the hydroxy group. Analysis of the HMBC spectrum completely supported the proposed location of the hydroxy group (Table 1). There were observed J^2 correlations between C-2 (δ_C 64.0) and the methylene protons of C-1 (δ_H 2.52) and C-3 (δ_H 3.07). Correlations of C-3 (δ_C 64.0) and the methyl protons of C-18 (δ_H 1.44) as well as J^3 correlations between the signal of C-1 (δ_H 50.9) and protons of carbons 3 (δ_H 3.07) and 20 (δ_H 1.19) were also observed. The most important HMBC and NOESY correlations for **2** are listed in Table 1. The structure of this compound was confirmed by X-ray crystallographic diffraction, using Cu Kα radiation, as *ent*-2α-hydroxy-16-oxo-17-norkauran-19-oic acid (**2**) (Figure 3). The configuration at C-2 was determined to be *S*.

Compound **2** was evaluated for its effect on radical and shoot growth of *L. sativa* (lettuce). Lettuce seeds, due to their ready availability and fast, complete, and uniform germination, are used commonly in allelopathic bioassays.[18] The choice of concentrations used and the duration of the experiment were based on previous work.[2,19] The results (Figure 4) showed almost an absolute predominance of a stimulatory activity, mainly in radical growth, where the stimulation attained ~30%, relative to the control, at 10^{-4} M. A slight inhibitory effect was observed for shoot growth at the same time.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a Perkin-Elmer 341 polarimeter. Infrared spectra were recorded on a Perkin-Elmer Spectrum One spectrometer. ^1H NMR and ^{13}C NMR spectra were obtained on a Bruker model DRX 400 Avance with standard pulse sequences operating at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR, respectively. Deuterated solvents and reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). The HRESIMS was recorded with ESI Q-TOFMicro (Micromass, UK) equipment. The

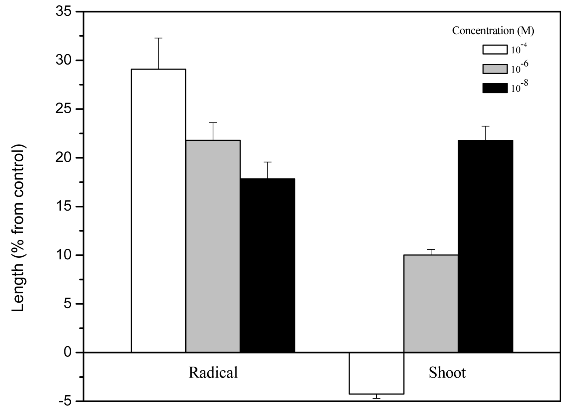

Figure 4. Effect of compound **2** on radical and shoot length of *Lactuca sativa*. Values are presented as percentage differences from the control, with zero representing an observed value identical to the control, a positive value representing stimulation, and a negative value representing inhibition.

data were collected from a sample diluted in methanol−TFA 0.1%. The standard conditions employed included vaporization temperature 80 °C, capillary voltage 1600 V, sample cone 35 V, and extraction cone 2.0 V. The data were analyzed using the Masslynx 4.0 software from Micromass for ESI experiments. Column chromatography was performed on silica gel (Merck, Darmstadt, Germany) (230−400 mesh). Reagents for culture medium preparation were acquired from Labsynth and Biobrás (Brazil) and Acumedia (Lansing, MI). Solvents used for chromatography (column and thin-layer) were purchased from Merck (Darmstadt, Germany).

Plant Material. The aerial parts of *Wedelia paludosa* (Asteraceae) (12.08 kg) were collected in Usiminas Garden, Belo Horizonte, Minas Gerais, Brazil, in August 2006. The sample was identified by Dr. T. S. M. Grandi, Department of Botany, UFMG, Brazil, and a voucher specimen (BHCB 19033) was deposited in the UFMG Biological Institute Herbarium (Brazil).

Substrate Preparation. To a suspension containing (1 g) *ent*-kaur-16-en-19-oic acid (kaurenoic acid), isolated from the aerial parts of *W. paludosa* (9.6% of ethanolic crude extract), were added 3.2 g (16.0 mmol) of $NaIO_4$ in 100 mL of THF−H_2O (1:1) and also a crystal of OsO_4. After overnight stirring at room temperature, workup ($NaHSO_3$ treatment) followed by flash column chromatography, 346 mg of *ent*-16-oxo-17-norkauran-19-oic acid (**1**) was obtained, mp 229−230 °C (lit. 232−234 °C).[20] This compound exhibited spectroscopic data consistent with literature values.[20]

Biotransformation Experiment. A pure culture of *F. proliferatum* (CML 287), from the Lavras mycological collection housed at the Universidade Federal de Lavras (UFLA, MG, Brazil), maintained in PDA under refrigeration, was added to two conical flasks with sterile liquid medium (200 mL/flask) containing (g/L) iron sulfate (0.01 g/L), magnesium sulfate (0.5 g/L), potassium chloride (0.5 g/L), potassium phosphate dibasic (1.0 g/L), sodium nitrate (3.0 g/L), and sucrose (30.0 g/L) in distilled water. The flasks were maintained under magnetic stirring at room temperature (~25 °C) for 4 days. Portions of this material (15 mL each) were transferred aseptically to 24 conical flasks containing fresh sterile medium and incubated under orbital stirring for 48 h.[6] Then, *ent*-16-oxo-17-norkauran-19-oic acid (**1**, 0.37 g) was added in chloroform (0.5 mL/flask; 79.8 mg/L). After 14 days, the flask contents were filtered using Whatman #1 filter paper for mycelia removal, and the broth was extracted with ethyl acetate. After solvent removal, this filtrate furnished a residue (3.32 g). This residue was column chromatographed on silica gel (215 g) using an elution gradient of hexane, CH_2Cl_2, EtOAc, and CH_3OH. A group of fractions eluted with 100% EtOAc (143.2 mg) exhibited white crystals and was further purified by passage over a silica gel column, from which a white solid (64.6 mg; 17.6% bioconversion) eluted with hexane−ethyl acetate (2:8) as thin, shiny needles. This compound was shown to be pure by TLC and was identified as *ent*-2α-hydroxy-16-oxo-17-norkauran-19-oic acid (**2**): white needles (CH_3OH); mp 261−265 °C; $[\alpha]^{25}_D$ 34.8 (*c* 0.75, MeOH); IR (KBr) ν_{max} 3496 (OH), 2941 (OH), 1711 (CO), 1243 (CH), 1191 (CH), 1024 (CH) cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) and

^{13}C NMR (C_5D_5N, 100 MHz), see Table 1; HRESIMS: *m/z* 343.3309 $[M + Na]^+$ (calcd for $C_{19}H_{28}O_4Na$, 343.4129).

Single-Crystal X-ray Analysis of 2. Compound **2** crystallized after slow evaporation of a saturated solution of MeOH as colorless prismatic crystals. Single-crystal X-ray diffraction data were collected at 292 K on an Oxford Gemini Atlas Ultra diffractometer with graphite-monochromated radiation; λ (Cu Kα) = 1.541838 Å using the CrysAlis-Pro data collection and data processing software. **2**: $C_{19}H_{28}O_4$, F_{000} = 1392, M_r = 320.41, orthorhombic, a = 7.8483(1) Å, b = 19.6010(6) Å, c = 21.7285(3) Å, V = 3342.59(12) Å^3, $P2_12_12_1$, D_x = 1.273 Mg m^{-3}, θ_{max} = 66.2°, Z = 8. The intensities of reflections were used to solve, by direct methods, and refine the structures on F^2 using the WINGX program. All hydrogen atoms were located from difference maps and included in the refinements as riding. Altogether 14 003 measured reflections and 5626 independent reflections were obtained, R_{int} = 0.029, 423 parameters refined, $R[F^2 > 2\sigma(F^2)]$ = 0.033, $wR(F^2)$ = 0.086, S = 1.04, $(\Delta/\sigma)_{max}$ = 0.001, $\Delta\rho_{min}$ = −0.17, $\Delta\rho_{max}$ = 0.23 e Å^{-3}, Flack parameter = 0.02(14). The absolute structure was determined, giving a Flack parameter of 0.01(15).[21]

Biological Assay. Compound **2** was evaluated on the growth of the radicals and shoots of *L. sativa* (lettuce) at concentrations of 10^{-4}, 10^{-6}, and 10^{-8} M, according to methodology previously described.[19] The effects on growth were expressed as percent differences from the controls and consisted of differences (in cm) between mean values of the seeds with test compound and the mean value for the controls (seeds without addition of test compound)/mean values for control × 100. Thus, zero represents the control, positive values represent stimulation of the studied parameter, and negative values represent inhibition. Gibberellic acid, used as control, stimulated radical growth at the lower concentration (∼90% stimulation), while the shoot growth was stimulated by this compound at all tested concentrations. Compound **2** was less active than the positive control (gibberellic acid) on the radical growth and presented a different behavior toward the shoots in relation to gibberellic acid, being an inhibitor at the higher tested concentrations and stimulating growth at lower concentrations.

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Supporting Information Available: NMR spectra of compound **2**. This information is available free of charge via the Internet at http://pubs.acs.org.

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(21) Crystallographic data for the structure reported in this paper have been deposited (number 777058) with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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