Cerinolactone, a Hydroxy-Lactone Derivative from *Trichoderma cerinum*

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Supporting Information

**ABSTRACT:** A novel metabolite, 3-hydroxy-5-(6-isopropyl-3-methylene-3,4,4a,5,6,7,8,8a-octahydronaphthalen-2-yl)dihydrofuran-2-one, trivially named cerinolactone (1), has been isolated from culture filtrates of *Trichoderma cerinum* together with three known butenolides containing the 3,4-dialkyfuran-2-(SH)-one nucleus, harzianolide (2), T39butenolide (3), and dehydroharzianolide (4). The structure of 1 was determined by spectroscopic methods, including UV, MS, and 1D and 2D NMR analyses. In vitro tests with the purified compound exhibited activity against *Pythium ultimum*, *Rhizoctonia solani*, and *Botrytis cinerea*.

Biological control involves the use of beneficial organisms and/or their products in order to reduce the negative effects of plant pathogens and promote beneficial effects to the plant. Thus far, numerous biocontrol agents (BCAs) have been registered as commercially available formulations containing various microbial antagonists, of the genera *Agrobacterium*, *Pseudomonas*, *Streptomyces*, *Bacillus*, *Gliocladium*, *Trichoderma*, *Ampelomyces*, and *Coniothyrium*.

The main mechanisms involved in the antagonistic interactions between BCAs and pathogenic fungi are (i) antibiosis with production of secondary metabolites; (ii) mycoparasitism or hyperparasitism; (iii) competition for nutrients; and (iv) competition for niche colonization. In addition to the direct negative effects to phytopathogens, some BCAs are also able to stimulate plant defense response to pest attack, promote seed germination and plant growth, increase nutrient availability, and improve crop production.

*Trichoderma* strains are among the most studied and applied fungal BCAs in industry and agriculture, and they secrete several secondary metabolites with different biological activities. The accumulation of these natural compounds depends on the species and/or the strain and the equilibrium between elicited biosynthesis and biotransformation rates or degradation by other microbes.

The composition of metabolic profiles (the “metabolome”) of *Trichoderma* species is complex because of the wide range of compounds produced and the molecular activities identified, including the recently determined role in the activation of plant resistance and growth promotion. Obviously, metabolomic studies may provide new insights on the mechanisms that regulate the complex interactions between plants, fungal phytopathogens, and microbial antagonists of the genus *Trichoderma*, thus improving the usefulness of these beneficial agents.

This paper describes the isolation and characterization of metabolites produced by *Trichoderma cerinum* strain CH296 isolated from avocado roots in Spain. This strain produces three known butenolides containing the 3,4-dialkyfuran-2-(SH)-one nucleus, which have already been reported from different strains of *T. harzianum*. We also describe here the production and structure of a novel hydroxy-lactone derivate (1).

The EtOAc extract of *T. cerinum* culture filtrate was fractionated by silica gel column chromatography (CC). Further preparative TLC separations yielded the three known butenolides harzianolide (2), dehydroharzianolide (4), and T39butenolide (3), as well as the new metabolite named herein cerinolactone (1). The structures of the known compounds were determined by comparison of their NMR spectroscopic data with those reported in the literature.

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In addition, an auxin-like activity was observed on etiolated pea (Pisum sativum) stems treated with harzianolide, which also affected the growth and the plant defense responses of tomato (Solanum lycopersicum) and canola (Brassica napus) seedlings. In particular, a reduction in disease symptoms was noted when tomato and canola seedlings were treated with harzianolide (at both 10 and 1 mg L\(^{-1}\)) and then inoculated with a spore suspension of Botrytis cinerea or Leptosphaeria maculans.

**Table 1.** \(^1\)H and \(^{13}\)C NMR Spectral Data of Cerinolactone (1) (in CDCl\(_3\))

<table>
<thead>
<tr>
<th>Position</th>
<th>(\delta_\text{H} (\text{in Hz}))</th>
<th>(\delta_\text{C} (\text{in ppm}))</th>
<th>HMBC</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>125.5 CH</td>
<td>5.91 br s</td>
<td>14, 9</td>
</tr>
<tr>
<td>2</td>
<td>134.9 qC</td>
<td>1a, 1b, 1, 4a, 4b</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>152.3 qC</td>
<td>10, 4a, 4b, 15a</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>36 CH(_3)</td>
<td>2.06(^a)</td>
<td>15a, 15b, 10, 5a, 5b</td>
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<tr>
<td>5</td>
<td>26.5 CH(_2)</td>
<td>1.22(^b)</td>
<td>11, 4a, 4b</td>
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<tr>
<td>6</td>
<td>46.5 CH</td>
<td>1.23(^b)</td>
<td>5b, 11, 12, 13, 7</td>
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<tr>
<td>7</td>
<td>24.5 CH(_2)</td>
<td>2.05(^b)</td>
<td>9, 8, 6</td>
</tr>
<tr>
<td>8</td>
<td>25.0 CH(_2)</td>
<td>2.07(^b)</td>
<td>1, 9, 10, 7</td>
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<tr>
<td>9</td>
<td>44.7 CH</td>
<td>1.67(^b)</td>
<td>10, 8a</td>
</tr>
<tr>
<td>10</td>
<td>44.0 CH</td>
<td>1.80 m</td>
<td>1, 15a, 15b, 4a, 4b, 9</td>
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<tr>
<td>11</td>
<td>26.4 CH</td>
<td>2.13 m</td>
<td>12, 13, 6</td>
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<td>12</td>
<td>21.5 CH(_2)</td>
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<td>13</td>
<td>15.2 CH(_2)</td>
<td>0.7 d (7.30)</td>
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</tr>
<tr>
<td>14</td>
<td>80.9 CH</td>
<td>5.01 br dd (3.80, 4.09)</td>
<td>1, 1a, 2′</td>
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<tr>
<td>15</td>
<td>103.7 CH(_2)</td>
<td>4.56 d (1.69)</td>
<td>4a, 4b, 10</td>
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<td>4.69 d (1.46)</td>
<td></td>
</tr>
<tr>
<td>1′</td>
<td>34.3 CH(_3)</td>
<td>2.38 m</td>
<td>2′</td>
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<tr>
<td></td>
<td></td>
<td>2.45 m</td>
<td></td>
</tr>
<tr>
<td>2′</td>
<td>67.4 CH</td>
<td>4.50 t (7.89)</td>
<td>1a, 1b</td>
</tr>
<tr>
<td>3′</td>
<td>177.1 qC</td>
<td>1a, 1b, 2′</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) All correlations represent two or three-bond couplings. \(^b\) Overlapping NMR signals. Abbreviation: s: singlet; d: doublet; t: triplet; dd: doublet of doublets; m: multiplet; dt: doublet of triplet; br: broad.

The connectivity of the spin systems was deduced by a long-range \(^1\)H–\(^{13}\)C heterocorrelated experiment that was obtained with the HMBC (Table 1). In particular, the C-3′ correlation with 2′-H, 1′-Ha, and 1′-Hb indicated the presence of a hydroxy-\(\gamma\)-lactone ring. The connection of the \(\gamma\)-lactone ring with the other parts of the molecule was revealed by the connection of C-2 with 1′-Ha and 1′-Hb, 1-H with 14-H, and 1-H with C-15. Moreover, connection between 1-H and C-9, C-10, C-8, and C-2 allowed us to correlate C-1 with C-9, C-9 with C-10, and C-9 with C-8. The C-4 and C-10 carbons were found correlated with 15-Ha and 15-Hb. The carbon C-6 was connected with 7-Ha and also showed correlations to the proton signals of two geminal methyls, 12-H and 13-H, which in turn were correlated with C-5. A vicinal correlation between H-1 (5.91 ppm) and H-9 (1.67 ppm) protons was suggested by the related COSY cross-peak. Moreover, according to the Karplus–Conroy relationship regarding the vicinal H–H coupling constants, the dihedral angle \(\phi\) occurring between these two protons is between 75° and 105° since the coupling constant extrapolated by the broadened H-1 signal in the protonic spectrum is slightly lower than 1.5 Hz. In addition, as shown in Figure 2, this conformation evidence was further confirmed by the low-intensity cross-peak observed in the NOESY spectrum correlating H-1 and H-9 protons.

The presence of the above structural features in 1 was verified by the correlation observed in the TOCSY, which implied that the structure of the metabolite is 3-hydroxy-5-(6-isopropyl-3-methylene-3,4,4a,5,6,7,8,8a-octahydronaphthalen-2-yl)dihydrofuran-2-one (1).

The configuration of cerinolactone was evident from the NOESY experiments on 1. The strong NOE correlation observed from the protons of the \(\gamma\)-lactone ring and the other part of the molecule led to the relative stereochemistry of 1 that

**Figure 1.** Structural fragments of cerinolactone (1).

**Figure 2.** Selected NOE correlations exhibited by cerinolactone (1).
was established unambiguously, as shown in Figure 2. Moreover, the MM-2 energy calculation of cerinolactone (21.6 kcal/mol) presented the most stable conformational model (Figure 2), which was completely in agreement with the NOE data.

To confirm the proposed structure, a sample of cerinolactone was acetylated using acetic anhydride/pyridine. The $^1$H NMR spectrum of the product contained one acetate resonance, confirming the presence of the hydroxyl group bound to the lactone ring. The $^{13}$C NMR spectrum contained an additional signal for the acetyl carbonyl resonance at 169.9 and for the acetyl methyl carbon at 20.1 ppm. Moreover, all the correlations observed for the acetyl cerinolactone (acetate methyl carbon at 20.1 ppm. Moreover, all the signal for the acetyl carbonyl resonance at 169.9 and for the conduct all liquid-state NMR measurements at a temperature of 25 °C, and 2400 transients, respectively, and a spectral width of 250 ppm (4.8015 kHz) as spectral width, whereas proton-decoupled carbon acquisitions were executed by both inverse-gated and DEPT pulse sequences, adopting 8 and 2 s of equilibrium delay, 12 500 ms (μs) delay, and 128 scans, respectively, while HSQC and HMBC experiments were optimized for 4.5 Hz short- and 10 Hz long-range $^{13}$C couplings, respectively. All executed 2D experiments were gradient enhanced, except for the TOCSY acquisition. The free induction decay of monodimensional spectra was multiplied by an exponential factor corresponding to 0.1 Hz, for $^1$H and $^{13}$C acquisitions, and to 1 Hz for the DEPT 135° experiment. All above-mentioned spectra were baseline corrected and processed by using both Bruker Topspin software (v.1.3) and MestReC NMR processing software (v.4.9.9.9).

Electrospray mass spectra were recorded on a Perkin-Elmer API 100 LC-MS, using a probe voltage of 5300 V and a declustering potential of 50 V. High-resolution MALDI-TOF MS analyses were carried out on a Perseptive Biosystems (Framingham, MA, USA) Voyager DE-PRO instrument equipped with a N$_2$ laser (337 nm, 3 ns pulse width). UV spectra were recorded with a UNICAM Helios Beta UV–vis. Column chromatography was performed using silica gel (Merck silica gel 60 GF254), and TLC with glass precoated silica gel GF254 plates (Merck Kieselgel 60 GF254, 0.25 mm). The compounds were detected on TLC plates using UV light (254 or 366 nm) and/or by dipping the plates in a 5% (v/v) H$_2$SO$_4$ solution in EtOH followed by heating at 110 °C for 10 min.

**Fungal Strains.** T. cerinum strain CH296 was isolated from the rhizosphere of avocado trees in Granada, southern Spain. This isolate was identified by the Centraalbureau voor Schimmelcultures—Fungal Biodiversity Centre, Institute of the Royal Netherlands Academy of Arts and Sciences (Utrecht, The Netherlands). The pathogens P. ultimum, R. solani, and B. cinerea were isolated from field crops in Italy. The antagonistic fungus T. cerinum and the R. solani were maintained on potato dextrose agar (PDA, Sigma, St Louis, MO, USA) slants at room temperature (rt) and subcultured biweekly.

**Liquid Culture and Metabolite Production.** Two 7 mm diameter plugs of T. cerinum, obtained from actively growing margins of PDA cultures, were inoculated in 5 L conical flasks containing 1 L of potato dextrose broth (one-fifths strength—Sigma). The stationary cultures were incubated for 21 days at 25 °C. The fungal cultures were vacuum filtered through filter paper (Whatman No. 4) to remove the biomass, and the culture filtrate was collected.

**Extraction and Isolation of T. cerinum Secondary Metabolites.** The culture filtrate (10 L) was acidified to pH 4 with 5 M HCl and extracted exhaustively with ethyl acetate. The combined organic extracts were dried (Na$_2$SO$_4$) and evaporated under reduced pressure at 35 °C. The yellow residue recovered was fractionated by column chromatography (silica gel; 200 g) eluted with a gradient of petroleum ether/Me$_2$CO, 7:3). The compounds were detected on TLC plates using UV light (254 or 366 nm) and/or by dipping the plates in a 5% (v/v) H$_2$SO$_4$ solution in EtOH followed by heating at 110 °C for 10 min.

**Cerinolactone.** 1H-13C HSQC (heteronuclear single-quantum correlation) and HMBC (heteronuclear multiple bond coherence).

The homonuclear and heteronuclear 2D experiments were acquired with 48 and 80 scans, respectively, 16 dummy scans, a time domain of 2k points (F2), and 256 experiments (F1). TOCSY and NOESY experiments were conducted with a mixing time of 80 and 65 ms, respectively, while HSQC and HMBC experiments were optimized for 145 Hz short- and 10 Hz long-range $^{13}$C couplings, respectively. All executed 2D experiments were gradient enhanced, except for the TOCSY acquisition. The free induction decay of monodimensional spectra was multiplied by an exponential factor corresponding to 0.1 Hz, for $^1$H and $^{13}$C acquisitions, and to 1 Hz for the DEPT 135° experiment. All above-mentioned spectra were baseline corrected and processed by using both Bruker Topspin software (v.1.3) and MestReC NMR processing software (v.4.9.9.9).

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yield the acetyl derivative 5 (1.4 mg, 49%). Acetyl cerinolactone 5: white, amorphous solid; UV (CHCl₃) λ_max (log ε) 240 (0.34) nm; [α]D +28 (CHCl₃ c 1.0); 1H NMR data for 5, selected peaks 2.16 (3H, s, MeCO), 5.38 (1H, m, H2′), 2.55 (1H, m, H1′), 2.38 (1H, m, H1′); 13C NMR data for 5, selected peaks 32.6 (–CH3), 169.9 (–C=O), 70.0 (–CH), 32.6 (–CH₂); HRMS (MALDI) m/z 333.1936 [M + H]+ (calcd for C₂₀H₂₈O₄ + H, 333.1988).

**Antifungal Assays.** The purified compound 1 was tested against the phytopathogenic agents *P. ultimum*, *R. solani*, and *B. cinerea*. The method described by Dunlop et al. was used with some modifications. Briefly, pathogen plugs (5 mm diameter) were placed at the center of 1/5 PDA Petri dishes. Then 10 μL of the purified compound was applied to the surface of each plug at concentrations ranging from 0.01 to 200 μg plug⁻¹. The controls were treated only with 10 μL of EtOAc go. The pathogen growth was evaluated daily by measuring the colony diameter (mm). Each treatment consisted of three replicates, and each experiment was repeated at least twice. The *Trichoderma* metabolite 6-pentyl-α-pyrone was used as positive control, since it inhibited completely *P. ultimum* and *R. solani* at 100 μg plug⁻¹ and *B. cinerea* at 150 μg plug⁻¹.

**Plant Growth Promotion Assay.** To test the plant growth promotion activity, tomato seeds were treated with different amounts of cerinolactone (1 μg to 0.1 ng per seed). The method described by Vinale et al. was used to determine the effect on seed germination and plant growth.

### ASSOCIATED CONTENT

**Supporting Information**

1H and 13C NMR, DEPT 135, COSY, HSQC, HMBC, TOCSY, and NOESY spectra of 1 and plant growth activity results are available free of charge via the Internet at http://pubs.acs.org.

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### REFERENCES


