JOURNAL OF NATURAL PRODUCTS

Note

pubs.acs.org/jnp

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

Francesco Vinale,*[,†,‡] Isabel Arjona Girona,[§] Marco Nigro,[†,‡] Pierluigi Mazzei,[⊥] Alessandro Piccolo,[⊥] Michelina Ruocco,[†] Sheridan Woo,[†,‡] David Ruano Rosa,[§] Carlos López Herrera,[§] and Matteo Lorito[†,‡]

[†]CNR—Istituto per la Protezione delle Piante (IPP-CNR), 80055 Portici, 80055 Naples, Italy

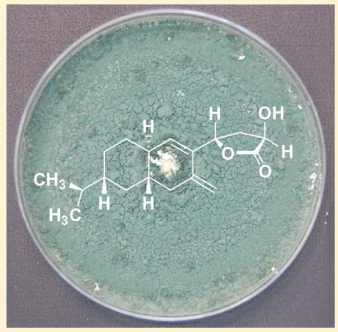
[‡]Dipartimento di Arboricoltura Botanica e Patologia Vegetale, Università degli Studi di Napoli "Federico II", Portici, 80055 Naples, Italy

[§]Instituto de Agricultura Sostenible CSIC, 14080 Córdoba, Spain

[⊥]Centro Interdipartimentale di Spettroscopia di Risonanza Magnetica Nucleare (CERMANU), Università degli Studi di Napoli '"Federico II", Portici, 80055 Naples, Italy

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-dialkylfuran-2(5*H*)-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.[1−5]

Trichoderma strains are among the most studied and applied fungal BCAs in industry and agriculture,[6] and they secrete several secondary metabolites with different biological activities.[7−9] 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.[9]

The composition of metabolic profiles (the "metabolome") of *Trichoderma* species is complex because of the wide range of compounds produced[4] and the molecular activities identified, including the recently determined role in the activation of plant resistance and growth promotion.[3,11−13] 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.[4]

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-dialkylfuran-2(5*H*)-one nucleus, which have already been reported from different strains of *T. harzianum*.[14−17] We also describe here the production and structure of a novel hydroxy-lactone derivate (**1**).

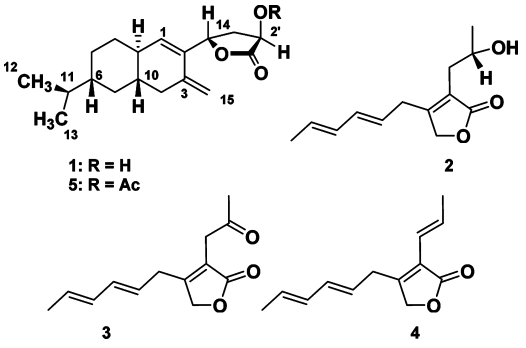
The EtOAc extract of *T. cerinum* culture filtrate was fractionated by silica gel column chromatography (CC). Further preparative TLC separations yielded the three known butenolides harzianolide (**2**),[14−17] dehydroharzianolide (**4**),[14] and T39butenolide (**3**),[15] as well as the new metabolite 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.[14−17] These butenolides were reported to be active against *Gaeumannomyces graminis* var. *tritici* and *Rhizoctonia solani*.[14,15]

Received: July 6, 2011
Published: December 23, 2011

dx.doi.org/10.1021/np200577t | *J. Nat. Prod.* 2012, 75, 103−106

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.[11] 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*.[11]

Scheme 1. Structural Formula of Compounds 1, 2, 3, 4, and 5



Cerinolactone (1 mg/L) was obtained as a white, amorphous solid. The ^{13}C NMR data (Table 1) and the molecular weight of 291.1854 revealed a molecular formula of $C_{18}H_{26}O_3$, consistent with six unsaturations. Four carbon signals at 103.7, 125.5, 134.9, and 152.3 ppm revealed two double bonds, whereas a carbon signal at 177.1 ppm indicated a γ-lactone ring. The other 13 signals of the ^{13}C spectrum were all shifted upfield in the 15.2−80.9 ppm range. The DEPT data demonstrated that one of the protons in the molecule is bound to oxygen and the presence of two CH_3, six CH_2, seven CH, and three fully substituted C atoms. The $^1H-^1H$ COSY and HSQC analyses plus the chemical shift evaluation permitted identification of the structural fragment of cerinolactone (Figure 1).

Table 1. ^1H and ^{13}C NMR Spectral Data of Cerinolactone (1) (in $CDCl_3$)

position	δ_C mult.	δ_H (J in Hz)	HMBC[a]
1	125.5 CH	5.91 br s	14, 9
2	134.9 qC		1′a, 1′b, 1, 9, 4a, 4b
3	152.3 qC		10, 4a, 4b, 15a
4	36 CH_2	2.06[b]	15a, 15b, 10, 5a, 5b
		2.41 m	
5	26.5 CH_2	1.22[b]	11, 4a, 4b
		1.15[b]	
6	46.5 CH	1.23[b]	5b, 11, 12, 13, 7
7	24.5 CH_2	2.05[b]	9, 8, 6
		1.50[b]	
8	25.0 CH_2	2.07[b]	1, 9, 10, 7
		1.64[b]	
9	44.7 CH	1.67[b]	10, 8a
10	44.0 CH	1.80 m	1, 15a, 15b, 4a, 4b, 9
11	26.4 CH	2.13 m	12, 13, 6
12	21.5 CH_3	0.9 d (7.02)	
13	15.2 CH_3	0.7 d (7.30)	
14	80.9 CH	5.01 br dd (3.80, 4.09)	1, 1′a, 2′
15	103.7 CH_2	4.56 d (1.69)	4a, 4b, 10
		4.69 d (1.46)	
1′	34.3 CH_2	2.38 m	2′
		2.45 m	
2′	67.4 CH	4.50 t (7.89)	1′a, 1′b
3′	177.1 qC		1′a, 1′b, 2′

[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.

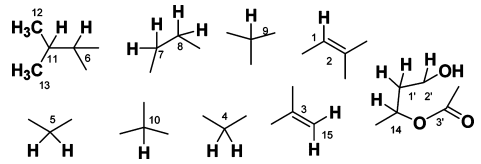


Figure 1. Structural fragments of cerinolactone (**1**).

The connectivity of the spin systems was deduced by a long-range $^1H-^{13}C$ heterocorrelated experiment that was obtained with the HMQC (Table 1). In particular, the C-3′ correlation with 2′-H, 1′-Ha, and 1′-Hb indicated the presence of a hydroxy-γ-lactone ring. The connection of the γ-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,[18] the dihedral angle ϕ 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.

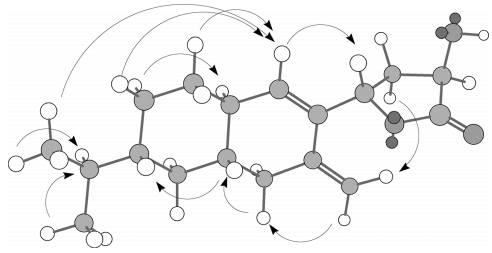


Figure 2. Selected NOE correlations exhibited by cerinolactone (**1**).

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 γ-lactone ring and the other part of the molecule led to the relative stereochemistry of **1** that

dx.doi.org/10.1021/np200577t | J. Nat. Prod. 2012, 75, 103−106

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 acetate methyl carbon at 20.1 ppm. Moreover, all the correlations observed for the acetyl cerinolactone (**5**) confirmed the structure of **1**.

In vitro assays demonstrated that the vegetative growth of the plant pathogen *P. ultimum* was completely inhibited using 10 μg of **1**, while applications less than 10 μg had a weak effect (1 μg, 24% inhibition, Figure 3). Moreover, 41% and 28%

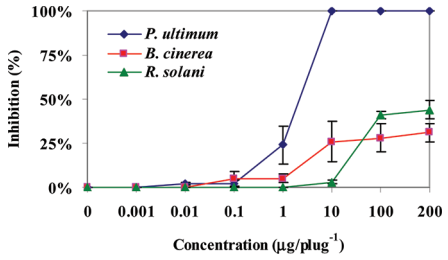


Figure 3. Antibiotic activity of **1**. (◇) *P. ultimum*; (Δ) *R. solani*; (□) *B. cinerea* (48 h of growth). Concentrations ranging from 0.001 to 200 μg plug^{-1}.

inhibition was registered using 100 μg of **1** against *R. solani* and *B. cinerea*, respectively (Figure 3). Growth of tomato seedlings was inhibited up to 63% and 70% (stem length) 3 days after treatment with **1** applied at concentrations of 1 and 0.1 ng per seed, respectively. However, **1** did not significantly alter the seedling growth 2 and 4 days after treatment.

EXPERIMENTAL SECTION

General Experimental Procedures. Isolated molecules were solubilized in 700 μL of deuterated chloroform (99.8% $CDCl_3$, Sigma Chemical Co.) and transferred into a stoppered NMR tube (5 mm, 7 in., 507-HP-7, NORELL), where the remaining void volume was gently degassed by a N_2 flux. Residual and deuterated solvent peaks were used as reference standards.

A 400 MHz Bruker Avance spectrometer, equipped with a 5 mm Bruker broad band inverse probe, working at the ^{1}H and ^{13}C frequencies of 400.13 and 100.61 MHz, respectively, was employed to conduct all liquid-state NMR measurements at a temperature of 25 ± 1 °C.

1D ^{1}H and ^{13}C acquisitions were conducted as follows: proton spectra were acquired with 2 s of thermal equilibrium delay, a 90° pulse length ranging between 7.95 and 8.1 μs, 50 transients, and 12 ppm (4.8015 kHz) as spectral width, whereas proton-decoupled carbon acquisitions were executed by both inverse-gated and DEPT 135° pulse sequences, adopting 8 and 2 s of equilibrium delay, 12 500 and 2400 transients, respectively, and a spectral width of 250 ppm (25.152 kHz). A time domain of 32 768 points was adopted for all cited monodimensional experiments.

Structural identification of secondary metabolites was achieved by means of 2D experiments: homonuclear ^{1}H−^{1}H COSY (correlation spectroscopy), TOCSY (total correlation spectroscopy), NOESY (nuclear Overhauser enhancement spectroscopy), and heteronuclear ^{1}H−^{13}C HSQC (heteronuclear single-quantum correlation) and HMBC (heteronuclear multiple bond coherence).

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 J_{CH} 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 GF_{254}), and TLC with glass precoated silica gel GF_{254} plates (Merck Kieselgel 60 GF_{254}, 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_2SO_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.[19] This isolate was identified by the Centraalbureau voor Schimmelcultures−Fungal Biodiversity Centre, Institute of the Royal Neteherlands 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 bimonthly.

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 sterile 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_2SO_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_2CO (1:0 to 0:1). Eleven fractions were collected and pooled on the basis of similar TLC profiles. Fraction 3 was further purified by preparative TLC (petroleum ether/Me_2CO, 8:2) to yield **4** (5 mg), while preparative TLC (petroleum ether/Me_2CO, 7:3) of fractions 4 and 6 yielded **2** (12 mg) and **3** (2 mg), respectively. UV, IR, ^{1}H NMR, ^{13}C NMR, and LC-MS of dihydroharzianolide (**4**), T39butenolide (**3**), and harzianolide (**2**) were identical to those reported by Almassi et al.[13] and Vinale et al.[14]

Successive preparative TLC of fraction 5 yielded **1** (10 mg) as a white, amorphous solid (R_f 0.5 by silica gel, eluent system petroleum ether/Me_2CO, 7:3).

Cerinolactone (1): white, amorphous solid; UV (MeOH) λ_{max} (log ε) 277 (0.13) nm; $[\alpha]_D$ +38 ($CHCl_3$; *c* 1.0); ^{1}H and ^{13}C NMR, see Table 1; ESMS (+) *m/z* 893 $[M_3 + Na]^+$, 603 $[M_2 + Na]^+$, 313 $[M + Na]^+$, 291 $[M + H]^+$; 273 $[M + H - H_2O]^+$; HRMS (MALDI) *m/z* 291.1854 $[M + H]^+$ (calcd for $C_{18}H_{26}O_3$ + H, 291.1882).

Acetylation of 1. Ac_2O (40 μL) was added to cerinolactone (**1**, 2.5 mg), dissolved in dry pyridine (80 μL). The mixture was stirred at rt for 2 h. After evaporation of excess reagent, the residue was purified by preparative TLC on silica gel (petroleum ether/Me_2CO, 2:8) to

dx.doi.org/10.1021/np200577t | *J. Nat. Prod.* 2012, 75, 103−106

yield the acetyl derivative **5** (1.4 mg, 49%). **Acetyl cerinolactone 5:** white, amorphous solid; UV ($CHCl_3$) λ_{max} (log ε) 240 (0.34) nm; $[\alpha]_D$ +28 ($CHCl_3$; *c* 1.0); ^{1}H 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′); ^{13}C NMR data for **5**, selected peaks 20.0 (−CH_3), 169.9 (−C═O), 70.0 (−CH), 32.6 (−CH_2); HRMS (MALDI) *m*/*z* 333.1936 [M + H]$^+$ (calcd for $C_{20}H_{28}O_4$ + 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.[20] 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. 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.[10] was used to determine the effect on seed germination and plant growth.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C 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.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +39 081 2539338. Fax: +39 081 2539339. E-mail: frvinale@unina.it.

■ ACKNOWLEDGMENTS

Work by F.V., M.R., S.W., and M.L. has been supported by the following projects: Calabria Region APQ Projects "S.Re.Va.P-r.O.", Mis. 124, Projects "CA.SVI.PR.OLI." and "MI.P.RE.-VEGE."; PRIN MIUR 2008 - prot. 2008SNPNC2 - prot. 2008WKPAWW; FIRB 2002 prot. RBNE01K2E7; PRIN 2003 prot. 2003070719-003; MIUR PON project no. DD12935 of 02/08/2002; MIUR PON project no. DD1219 of 05/10/2004; MIUR PON project no. DD1801 of 31/12/2004; EU TRICHOEST QLK3-2002-02032; EU 2E-BCA2. Work by I.A.G., D.R.R., and C.L.H. has been supported by the Spanish Plan Nacional I+D+I Grant AGL 2008-05453-C02-02/AGR. The authors thank A. Ritieni and R. Ferracane for the LC-MS, and F. Addeo and S. Cairo for MALDI-TOF analysis.

■ REFERENCES

(1) Harman, G. E.; Howell, C. R.; Viterbo, A.; Chet, I.; Lorito, M. *Nat. Rev. Microbiol.* **2004**, *2*, 43−56.
(2) Lorito, M.; Woo, S.; Iaccarino, M.; Scala, F. In *Microrganismi Antagonisti*; Iaccarino, M., Ed.; Idelson-Gnocchi s.r.l.: Naples, 2006; pp 146−175.
(3) Vinale, F.; Sivasithamparam, K.; Ghisalberti, E. L.; Marra, R.; Woo, S. L.; Lorito, M. *Soil Biol. Biochem.* **2008**, *40*, 1−10.
(4) Lorito, M.; Woo, S. L.; Harman, G. E.; Monte, E. *Annu. Rev. Phytopathol.* **2010**, *48*, 395−417.
(5) Vinale, F.; Ghisalberti, E. L.; Flematti, G.; Marra, R.; Lorito, M.; Sivasithamparam, K. *Lett. Appl. Microbiol.* **2010**, *50*, 380−385.
(6) Harman, G. E.; Kubicek, C. P. *Trichoderma and Gliocladium*, Vol II; Taylor & Francis: London, 1998; pp 1−379.
(7) Sivasithamparam, K.; Ghisalberti, E. L. In *Trichoderma and Gliocladium*; Harman, G. E., Kubicek, C. P., Eds.; Taylor and Francis Ltd: London, 1998; Vol. *1*, pp 139−191.
(8) Reino, J. L.; Guerrero, R. F.; Hernández-Galán, R.; Collado, I. G. *Phytochem. Rev.* **2008**, *7*, 89−123.
(9) Ghisalberti, E. L.; Rowland, C. Y. *J. Nat. Prod.* **1993**, *56*, 1799−1804.
(10) Vinale, F.; Ghisalberti, E. L.; Sivasithamparam, K.; Marra, R.; Ritieni, A.; Ferracane, R.; Woo, S. L.; Lorito, M. *Lett. Appl. Microbiol.* **2009**, *48*, 705−711.
(11) Vinale, F.; Sivasithamparam, K.; Ghisalberti, E. L.; Marra, R.; Barbetti, M. J.; Li, H.; Woo, S. L.; Lorito, M. *Physiol. Mol. Plant Pathol.* **2008**, *72*, 80−86.
(12) Vinale, F.; Flematti, G.; Sivasithamparam, K.; Lorito, M.; Marra, R.; Skelton, B. W.; Ghisalberti, E. L. *J. Nat. Prod.* **2009**, *72*, 2032−2035.
(13) Viterbo, A.; Wiest, A.; Brotman, Y.; Chet, I.; Kenerley, C. *Mol. Plant Pathol.* **2007**, *8*, 737−746.
(14) Almassi, F.; Ghisalberti, E. L.; Narbey, M. J.; Sivasithamparam, K. *J. Nat. Prod.* **1991**, *54*, 396−402.
(15) Vinale, F.; Marra, R.; Scala, F.; Ghisalberti, E. L.; Lorito, M.; Sivasithamparam, K. *Lett. Appl. Microbiol.* **2006**, *43*, 143−148.
(16) Ordentlich, A.; Wiesman, Z.; Gottlieb, H. E.; Cojocaru, M.; Chet, I. *Phytochemistry* **1992**, *31*, 485−486.
(17) Claydon, N.; Hanson, J. R.; Truneh, A.; Avent, A. G. *Phytochemistry* **1991**, *30*, 3802−3803.
(18) Breitmaier, E. *Structure Elucidation by NMR in Organic Chemistry - A Practical Guide*; John Wiley & Sons Ltd: England, 1993; pp 13−42.
(19) Ruano-Rosa, D.; Moral-Navarrete, L.; López-Herrera, C. J. *Span. J. Agric. Res.* **2010**, *8*, 1084−1097.
(20) Dunlop, R. W.; Simon, A.; Sivasithamparam, K.; Ghisalberti, E. L. *J. Nat. Prod.* **1989**, *52*, 67−74.