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Cremenolide, a new antifungal, 10-member lactone from *Trichoderma cremeum* with plant growth promotion activity

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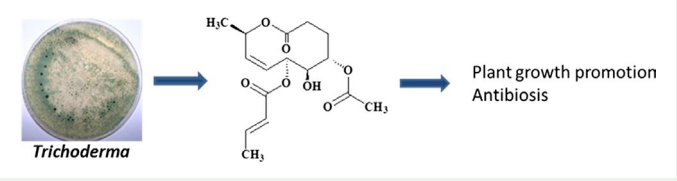
ABSTRACT

Trichoderma based products are considered an alternative to synthetic pesticides and fertilizers. These *Trichoderma* spp. are among the most studied and applied fungal BCAs in industry and agriculture and are known to secrete several secondary metabolites with different biological activities. The analysis 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 to biotic and abiotic stresses and growth promotion. A new 10-member lactone, but-2-enoic acid 7-acetoxy-6-hydroxy-2-methyl-10-oxo-5,6,7,8,9,10-hexahydro-2H-oxecin-5-yl ester, named cremenolide (**1**), has been isolated from culture filtrates of *Trichoderma cremeum*. The structure of cremenolide was determined by spectroscopic methods, including UV, MS, and 1D and 2D NMR analyses. *In vitro* tests showed that the purified compound inhibited the radial mycelium growth of *Fusarium oxysporum*, *Botrytis cinerea* and *Rhizoctonia solani*, and exerted a significant promotion of growth of tomato seedlings.



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1. Introduction

Fungi of the genus *Trichoderma* have been extensively studied, and are used as bioactive components of biopesticides and biofertilisers (Woo et al. 2014). They have been applied

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This work is dedicated to the memory of Prof Emilio L. Ghisalberti for his studies on *Trichoderma* secondary metabolites.
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for their beneficial effects on treated plant that include: antagonism of plant pathogens by using a variety of mechanisms (mycoparasitism or hyperparasitism; antibiosis; competition; cell wall-lytic enzyme activity) in addition to enhancement of plant growth, acquisition of soil nutrients and induction of plant defense responses (Harman 2000; Howell 2003; Harman et al. 2004; Marra et al. 2006; Vinale et al. 2008). The genus *Trichoderma* are well known producers of secondary metabolites that are biologically active on plants and fungal pathogens (Sivasithamparam & Ghisalberti 1998; Reino et al. 2008; Vinale et al. 2014; Ahluwalia et al. 2015). The production of *Trichoderma* secondary metabolites is strain dependent and varies in relation to the equilibrium between elicited biosynthesis and biotransformation rates (Vinale, Ghisalberti, et al. 2009). In the search for new 'effectors' with antibiotic and plant growth promotion activities among *Trichoderma*, we have studied the metabolites of *Trichoderma cremeum*. From the ethyl acetate extracts of the culture filtrate, we have isolated a new 10-member lactone, but-2-enoic acid 7-acetoxy-6-hydroxy-2-methyl-10-oxo-5,6,7,8,9,10-hexahydro-2H-oxecin-5-yl ester, named cremenolide (**1**). This compound inhibited the mycelium radial growth of *Fusarium oxysporum*, *Botrytis cinerea* and *Rhizoctonia solani*, and promoted growth of tomato seedlings.

2. Results and discussion

The culture filtrate of *T. cremeum* was extracted with ethyl acetate and the residue recovered was fractionated by silica gel column chromatography (CC). Fraction 2 obtained 312 mg of a new metabolite named herein cremenolide (**1**), that represents the major compound of the crude extract. Cremenolide (**1**) (32 mg/L) was a colourless solid with a molecular ion $[M]^+$ at 327.1416 and 16 carbons (^{13}C NMR coupled with DEPT spectra). With these data, the molecular formula was estimated to be $C_{16}H_{22}O_7$.

Four carbon signals at δ_c 128.56, 130.50, 145.88 and 122.01 revealed two double bonds, whereas a carbon signal at 174.99 ppm indicated a lactone ring. Signals at 169.98 and 165.38 indicated the presence of two ester carbonyls. The other nine signals of the ^{13}C spectrum were all shifted up field in the 16.64–78.5 ppm range. The DEPT data demonstrated the presence of three methyl at δ_c 16.64, 17.96 and 21.15, two methylenes at δ_c 23.86 and 32.61, four methines at δ_c 70.53, 71.63, 77.17 and 78.50 and that one of the protons in the molecule is bound to oxygen ($3CH_3$, $2CH_2$, 8CH, and 3 fully substituted C atoms). ^1H-^1H COSY, ^1H-^{13}C HSQC spectra and the chemical shift evaluation allowed identification of the cerinolactone fragments (Figure 1).

The connectivity of the fragments was deduced by a long-range ^1H-^{13}C heterocorrelated experiment which was obtained with the HMBC. The correlation of C-1 with 9-H, 2-Ha, 2-Hb, 3-Ha, 3-Hb and of C-4 with 2-Ha, 2-Hb, 3-Ha, 3-Hb and 5-H indicated the presence of a 10-member lactone ring. The key cross peaks of C-4 with 2″-H, C-1″ with 4-H and C-1′ with 6-H allowed us to correlate the other two remaining spin systems to the lactone ring. Due to the chemical shift and to complete the number of hydrogen the C-5 is bounded with an –OH.

The structure of **1** was consistent with the observed correlations observed in the TOCSY which implied that the structure of the metabolite is But-2-enoic acid 7-acetoxy-6-hydroxy-2-methyl-10-oxo-5,6,7,8,9,10-hexahydro-2H-oxecin-5-yl ester (**1**). The structure was also confirmed by LC-MS/MS analysis.

The configuration of cremenolide was evident from the NOESY experiments on **1**. The NOE correlation observed from the protons of the lactone ring and the methyl C-10 led to the relative stereochemistry of (**1**) that was established unambiguously, as shown in Figure 1.

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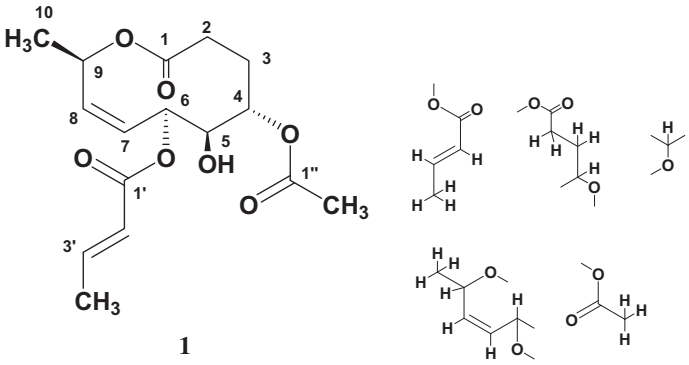

Figure 1. Structure of compound **1** (cremenolide) from *T. cremeum* and its structural fragments.

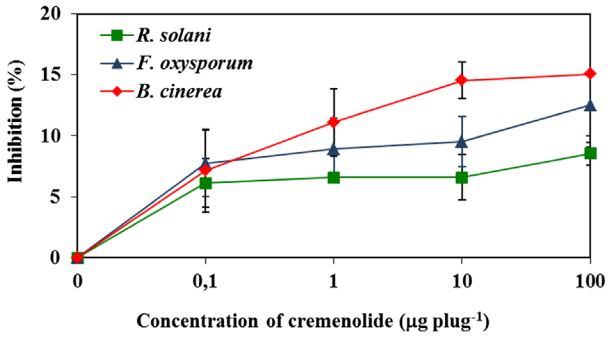

Figure 2. Antifungal activity of cremenolide against *F. oxysporum*, *B. cinerea* and *R. solani*. Concentrations ranged from 1 to 100 µg plug^{-1}.

NOESY also permitted to confirm the assignment performed for 5-H peak (4.5 ppm). Despite the apparent leaking of multiplicity, due to the minimised ^{1}H-^{1}H coupling resulting from the unfavourable dihedral angle formed with protons H-4 and H6, this peak was indeed vicinally coupled with these protons, as proved by TOCSY correlations and its relatively broadened shape. Moreover, the MM-2 energy calculation of cremenolide (41.253 kcal/mol) presented the most stable conformational model that was completely in agreement with the NOE data.

Cremenolide (**1**) significantly inhibited, in a concentration-dependent manner, the mycelial growth of *F. oxysporum*, *B. cinerea* and *R. solani*. In particular, the most significant effects were registered on *B. cinerea* at 10 µg plug^{-1} (15% of inhibition - Figure 2). Tomato seedlings assays showed that (**1**) promotes plant growth in terms of root length and fresh weight at 10 and 1 ppm (no effect at 0.1 and 0.01 ppm). Moreover, no significant effect was registered on seedling height at all concentrations used (Figure 3).

10-Member lactones belonging to the polyketides class, have been recently isolated by Malmierca and coauthors (2015) from a *tri5* gene-disrupted mutant of *Trichoderma arundinaceum* that lacks Harzianum A production. This microbe overproduced aspinolides B and C, which were not detected in the wild-type strain, and produced four new aspinolides (D–G). Aspinolide C had antibiotic effect against *B. cinerea* and *Fusarium sporotrichioides*, while aspinolide B did not show any activity against these two plant pathogens (Malmierca et al.

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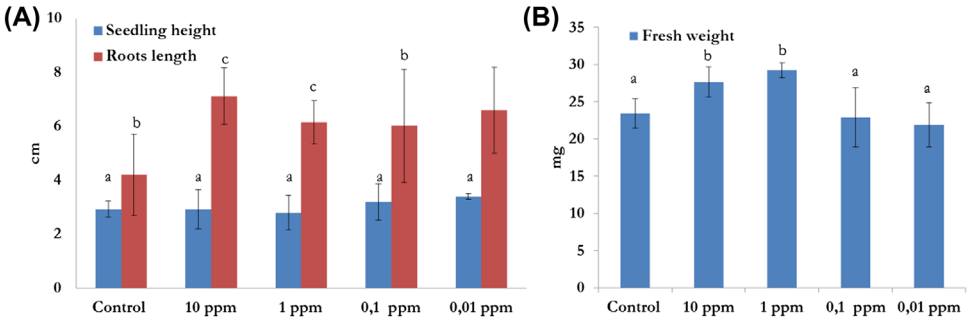

Figure 3. Effect of cremenolide on tomato growth measured as seedling height, root lengths (A) and fresh weight (B), 6 days after treatment. Values are means of 40 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly ($p < 0.05$).

2015). Aspinolides A–C were obtained also from the cultures of *Aspergillus ochraceus* but the antifungal activity of aspinolide A was not tested (Sun et al. 2012).

The treatment of tomatoes seedlings with 50 µg ml^{-1} of aspinolide B resulted in the proliferation of lateral roots, while aspinolide C caused a significant reduction in the number of lateral roots and seedling height as compared to control (Malmierca et al. 2015). Furthermore, higher concentration of aspinolide C (250 µg ml^{-1}) caused a strong reduction in development of the plant. Finally, Aspinolide C induced expression of tomato SA-related genes, which indicates that it may play a role in activation of tomato defence-related genes (Malmierca et al. 2015).

3. Experimental

3.1. General experimental procedures

CC was performed on silica gel (Merck silica gel 60 GF_{254}), and TLC with glass pre-coated 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 spraying the plates with 5% (v/v) H_2SO_4 solution in EtOH followed by heating at 110 °C for 10 min.

Electrospray mass spectra were recorded on a 6540 accurate-mass Q-TOF LC-MS instrument with a 1200 SL series HPLC (Agilent Technologies, Santa Clara, USA). The QTOF-MS instrument was operated with an electrospray ion source in positive ionisation mode.

UV spectra were recorded on a Jasco V-730 (ST) spectrophotometers (28600 Mary's Court, Easton, MD 21601).

Isolated molecules were solubilised in 1000 µL of deuterated chloroform (99.8% $CDCl_3$ – *Sigma Chemical Co*) and transferred into a stoppered NMR tube (5 mm, 7″, 507-HP-7 – *NORELL*) where remaining void volume was gently degassed by a N_2 flux. Peak of chloroform was used as reference to calibrate both ^{1}H and ^{13}C axes.

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 298 ± 1 K.

1D ^{1}H and ^{13}C acquisitions were conducted as follows: proton spectrum was acquired with 2 s of thermal equilibrium delay, a 90° pulse length ranging between of 8.65 µs, 64 transients and 16 ppm (6410.26 kHz) as spectral width, whereas proton-decoupled carbon

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acquisitions were executed by both inverse gated and DEPT 135° pulse sequences, adopting 7 and 4 s of equilibrium delay, respectively, as well as 12000 transients and a spectral width of 250 ppm (25.152 kHz). A time domain of 32768 points was adopted for all cited mono-dimensional experiments.

Structural identification of the secondary metabolite was achieved by means of 2D experiments: homo-nuclear ^{1}H-^{1}H COSY (Correlation Spectroscopy), TOCSY (Total Correlation Spectroscopy), NOESY (Nuclear Overhauser Enhancement Spectroscopy), and hetero-nuclear ^{1}H–^{13}C HSQC (Hetero-nuclear Single-Quantum Correlation) and HMBC (Hetero-nuclear Multiple Bond Coherence).

Homo-nuclear and hetero-nuclear 2D experiments were acquired with 48 scans, 16 dummy scans, a time domain of 2 k points (F2) and 256 experiments (F1). Moreover, TOCSY and NOESY experiments were conducted with a mixing time of 80 and 800 ms, respectively, while HSQC and HMBC experiments were optimised accounting for a short and long-range J_{CH} coupling of 145 and 10 Hz, respectively. All of 2D experiments were gradient enhanced, except for TOCSY and NOESY experiments.

The free induction decay of mono-dimensional spectra was multiplied by an exponential factor corresponding to 0.1 and 1 Hz for ^{1}H and ^{13}C 1D experiments, respectively. Each spectrum was baseline corrected and processed by using both Bruker Topspin Software (v.1.3) and MestReC NMR Processing Software (v.4.9.9.9).

3.2. Fungal strains

T. cremeum strain 506 was isolated from decaying wood in Central Poland (Warsaw). This isolate was identified by molecular identification with primers for the ITS1, ITS2 rRNA (internal transcribed region 1 and 2 of the rRNA gene cluster) and tef1 (translation elongation factor 1-alpha) sequences. The pathogens *F. oxysporum*, *B. cinerea* and *R. solani* were isolated from field crops in Italy. *T. cremeum*, *F. oxysporum* and *R. solani* were maintained on potato dextrose agar (PDA, High Media Pvt. Ltd., Mumbai–India) slants at room temperature and sub-cultured bimonthly. *B. cinerea* was maintained on malt extract peptone (MEP, SIGMA, St. Louis, MO., USA) slants at room temperature and subcultured bimonthly.

3.3. Liquid culture, extraction and metabolite isolation

Ten 7 mm diameter plugs of *T. cremeum*, from actively growing margins of PDA cultures, were inoculated in a 5 L conical flasks containing 1 L of sterile potato dextrose broth (PDB – High Media). The stationary cultures were incubated for 25 days at 25 °C, then the biomass was removed by filtration and the culture filtrate was collected.

The culture filtrate (10 L) was acidified to pH 4 with 5 M HCl and extracted exhaustively three times with EtOAc to obtain the crude extract (dried with Na_2SO_4; solvent evaporated under reduced pressure at 35 °C). The brown residue recovered (1.007 g) was fractionated by CC (silica gel; 300 g) eluted with EtOAc. Twelve fractions were collected and pooled on the basis of similar TLC profiles. Fraction 2, yielded **1** (301,7 mg) a colourless solid (*Rf* 0.6 by silica gel, eluent system CH_2Cl_2/MeOH 98:2 v:v).

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3.3.1. Cremenolide (1)

Colourless solid ($CHCl_3$), $[\alpha]_D$ +46 (*c* 0.18, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 258 (2.27) nm; ^1H (in $CHCl_3$) δ = 2.46, 2.13 m, 2.44, 1.64 m, 4.74 dt, 4.5 s, 4.96 m, 5.62, 5.63 br s, 5.06 m, 1.3 d, 5.82 dd (*J* = 1.56, 5.09), 6.9 dd (*J* = 1.55, 7.05), 1.86 dd (*J* = 7.05, 7.4), 2.06 s ppm; ^{13}C NMR (in $CHCl_3$) δ = 174.99 qC, 32.61 CH_2, 23.86 CH_2, 77.17 CH, 70.49 CH, 78.50 CH, 130.50 CH, 128.56 CH, 71.63 CH, 16.64 CH_3, 165.38 qC, 122.01 CH, 145.88 CH, 17.96 CH_3, 169.98 qC, 21.15 CH_3.

HR-ESI-MS: 675.2591 $[M_2 + Na]^+$; 365.0969 $[M + K]^+$; 349.1235 $[M + Na]^+$; 327.1416 $[M + H]^+$ (Calcd 326.1366); 344.1683 $[M + NH_4]^+$; 267.1212 $[M + H–CH_3COOH]^+$; 309.1313 $[M + H–H_2O]^+$; 241.1068 $[M + H–CH_3–CH=CH–COOH]^+$. HR-ESI-MS/MS: 181.0854 $[M + H–CH_3–CH=CH–COOH–CH_3COOH]^+$; 163.0754 $[M + H–CH_3–CH=CH–COOH–CH_3COOH–H_2O]^+$; 135.0803 $[M + H–CH_3–CH=CH–COOH–CH_3COOH–H_2O–CH_2–CH_2]^+$; 121.0647 $[M + H–CH_3–CH=CH–COOH–CH_3COOH–H_2O–CH_2–CH_2–CH_2]^+$; 107.0858 $[M + H–CH_3–CH=CH–COOH–CH_3COOH–H_2O–CH_2–CH_2–CH_2–CH_2]^+$; 93.0285 $[M + H–CH_3–CH=CH–COOH–CH_3COOH–H_2O–CH_2–CH_2–CH_2–CH_2–CH_2]^+$.

3.4. Antifungal assays

The purified compound **1** was tested against the fungal phytopathogenic agents *F. oxysporum*, *B. cinerea* and *R. solani*. The method described by Dunlop et al. (1989) was used with some modifications. Briefly, pathogen plugs (5 mm diameter) were placed at the centre of 1/5 PDA Petri dishes. Ten microliters of the purified compound was applied to the surface of each plug at concentrations ranging from 0.1 to 100 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 three times.

3.5. Plant growth promotion assay

To test the plant growth promotion activity, tomato seeds (*Lycopersicum esculentum* cv. San Marzano 823) were treated (coating) with different amounts of cremenolide (10–0.01 ppm corresponding to 100–0.1 ng per seed). The method described by Vinale, Flematti, et al. (2009) was used to determine the effect on seed germination and plant growth.

4. Conclusion

These results indicated that cremenolide can act as an antifungal compounds produced in the *Trichoderma*-pathogen interaction and as a plant growth promoters. Such compounds that affect plant metabolism or that can play a role in the environmental interactions established by beneficial microbes with other organisms present in the rhizosphere, may help the plant host to overcome the challenges from deleterious pathogens in agricultural production and help development of new biopesticides and biofertilisers formulations based on the bioactive metabolites.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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