Harzianic Acid, an Antifungal and Plant Growth Promoting Metabolite from *Trichoderma harzianum*

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Received September 7, 2009

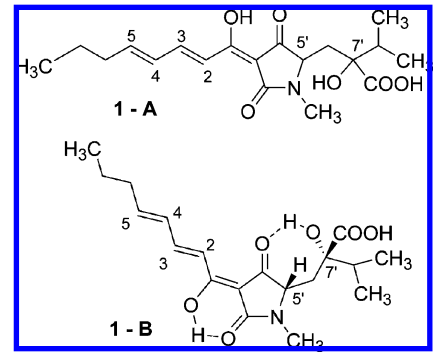
A *Trichoderma harzianum* strain, isolated from composted hardwood bark in Western Australia, was found to produce a metabolite with antifungal and plant growth promoting activity. The structure and absolute configuration of the fungal compound, harzianic acid (**1**), were determined by X-ray diffraction studies. Harzianic acid showed antibiotic activity against *Pythium irregulare*, *Sclerotinia sclerotiorum*, and *Rhizoctonia solani*. A plant growth promotion effect was observed at low concentrations of **1**.

The use of new products based on biocontrol agents and/or their metabolites for disease control is one of the most promising ways to reduce the dependence on synthetic pesticides in agriculture.[1] Various biocontrol agents have been registered and are available as commercial products, including strains belonging to the genus *Trichoderma*.[2] *Trichoderma* species have long been recognized as biological control agents (BCAs) for the control of plant diseases and for their ability to increase plant growth and development.[3−5] These fungi are known to be involved in complex interactions with host plants and resident microbial communities.[6,7] The mechanisms involved in the antagonism of *Trichoderma* toward phytopathogenic fungi include competition, antibiosis, direct mycoparasitism, and induction of systemic resistance to pathogens *in planta*.[4,7−10]

Fungal strains of the genus *Trichoderma* are well-known producers of secondary metabolites with antibiotic activity.[10−12] Their production varies in relation to (i) the specific compound; (ii) the strain and the species; (iii) the presence of other microbes; and (iv) the balance between elicited biosynthesis and biotransformation rates.[13] The involvement of secondary metabolites in the ability of *Trichoderma* spp. to activate plant defense mechanisms and regulate plant growth has recently been investigated.[14]

In the present study we investigated the metabolites produced by a strain of *Trichoderma harzianum*. This strain, isolated from composted hardwood bark in Western Australia, was suppressive versus the phytopathogenic agent *Pythium irregulare*. The fungus produced mainly harzianic acid (**1**), an incompletely characterized tetramic acid derivative.[15] Herein we report the isolation, the complete structural determination, and the antifungal and plant growth promoting activities of **1**.

Filtered culture broth of *T. harzianum* was extracted exhaustively with ethyl acetate. The red residue recovered was subjected to flash column chromatography (silica gel) with gradient elution, which resulted in 12 distinct fractions. Preparative TLC of fraction 7 yielded a small amount of a mixture of the known compounds 1-hydroxy-3-methylanthraquinone and 1,8-dihydroxy-3-methylanthraquinone. These molecules showed chromatographic and spectroscopic properties similar to standard samples.[16] In previous work the anthraquinones showed weak inhibition of *Gaeumannomyces graminis* var. *tritici*, but no activity against *Rhizoctonia solani* or *P. ultimum*.[17]



Fraction 3 consisted of crystalline material that showed the same ^{1}H and ^{13}C parameters as those of harzianic acid, a tetramic acid derivative (**1-A**). The structure proposed for this compound[15] was based on spectroscopic evidence, and no indication of the configuration of the stereogenic carbons was obtained. In order to characterize this metabolite, a single-crystal X-ray diffraction study was carried out, and the results are summarized below.

The structure and the absolute configuration of harzianic acid were determined to be as shown in Figure 1. Cu Kα radiation was used in the X-ray structure determination, and the Flack parameters were satisfactory for the quoted absolute stereochemistry. The chiralities of **1** at C(5′) and C(7′) are both *S* (**1-B**). There are intramolecular H-bonds between hydroxyl groups and carbonyl O atoms, i.e., between H(1) and O(2′) and between H(7′) and O(4′) (Figure 1). There is also an intramolecular H-bond between the carboxyl acid H(13′) and O(7′). An intermolecular H-bond from H(13′) to O(2′) in the molecule translated by one unit cell forms a 1D H-bonded polymer perpendicular to the *b* axis. Geometrical details are listed in Table 1 with the H-bonded polymer depicted in Figure 2.

Harzianic acid has also been isolated from liquid cultures of a fungal strain obtained from a soil sample in Amagi, Japan.[18] Co-occurring with this were the corresponding *N*-demethyl analogue

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10.1021/np900548p CCC: $40.75
Published on Web 11/06/2009

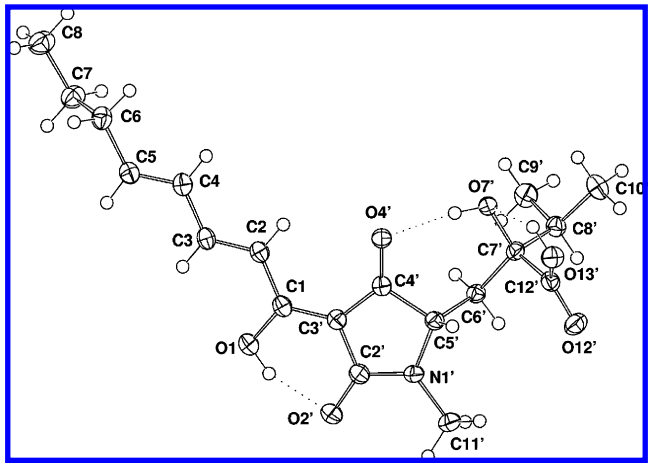

Figure 1. Molecular structure of harzianic acid (**1**) showing the numbering scheme and the intramolecular H-bonds.

Table 1. Hydrogen Bonds for **1** [Å and deg][a]

D−H···A	*d*(D−H)	*d*(H···A)	*d*(D···A)	∠(DHA)
O(1)−H(1O)···O(2′)	0.87(3)	1.80(3)	2.6113(15)	154(2)
O(7′)−H(7′O)···O(4′)	0.84(2)	1.87(2)	2.7018(13)	167.8(18)
O(13′)−H(13′)···O(7′)	0.86(2)	2.05(2)	2.5674(14)	117.5(18)
O(13′)−H(13′)···O(2′)[i]	0.86(2)	2.24(2)	3.0064(14)	147.8(19)

[a] Symmetry transformations used to generate equivalent atoms: i 1 *x*+1, *y*, *z*+1.

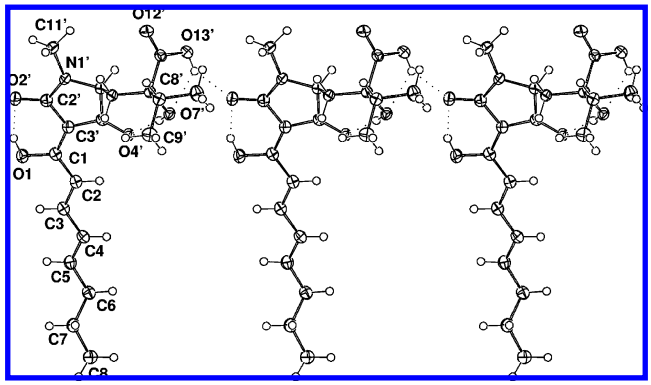

Figure 2. Diagram of harzianic acid (**1**) showing the intra- and intermolecular H-bonding scheme.

and a homologue in which the isopropyl group at C-7′ is replaced by a *sec*-butyl group.

Tetramic acids, or pyrrolidinediones, are widely distributed in nature and have been isolated from bacteria, myxomycetes, and sponges, as well as from marine and terrestrial fungi.[19,20] From a biosynthetic perspective, naturally occurring tetramic acids can be regarded to arise from the assembly of an amino acid and an activated acyl entity.[20,21] It is worth noting that examples in which L- or D-amino acids are involved, leading to 5*S*- or 5*R*-configuration, have been described. In some cases, these compounds were isolated as the calcium or magnesium salts and have been found to sequester calcium from silica gel.[22]

In preliminary experiments it had been established that harzianic acid affected the growth of cucumber plants, in the laboratory and in glasshouse conditions, by reducing disease severity caused by *Pythium* spp. and by promoting plant growth.[23]

In the present work, plate antifungal assays with 10 μg of **1** completely inhibited the growth of the phytopathogenic agents *P. irregulare* and *Sclerotinia sclerotiorum* (Figure 3). Moreover, 62% and 51% inhibition was registered using 1 μg of the *Trichoderma* metabolite against *P. irregulare* and *S. sclerotiorum*, respectively

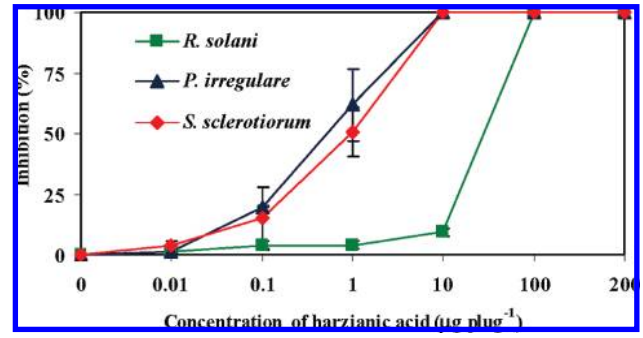

Figure 3. Antibiotic activity of **1**: (◇) *S. sclerotiorum*; (△) *P. irregulare*; (□) *R. solani* (48 h of growth). Concentrations ranged from 0.01 to 200 μg plug^{-1}.

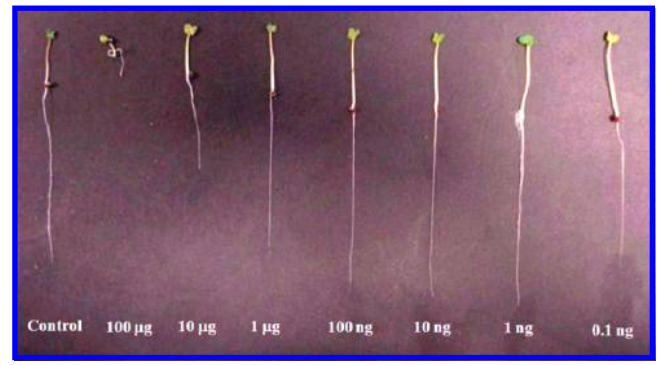

Figure 4. Canola seedlings (whose seeds were coated with different amounts of **1**) grown in water agar (WA) plates 10 days after sowing. From left: control, 100 μg, 10 μg, 1 μg, 100 ng, 10 ng, 1 ng, and 0.1 ng of harzianic acid per seed.

Table 2. Growth Promotion of Canola Seedlings Treated with Different Amounts of **1**[a]

	stem length (cm)	SD	root length (cm)	SD
control	2.30 a	0.34	5.98 a	1.18
100 μg	1.27 b	0.25	1.12 b	0.29
10 μg	1.55 b	0.37	3.30 c	1.01
1 μg	2.38 a	0.59	5.57 a	0.67
100 ng	3.27 c	0.39	6.33 a	0.38
10 ng	3.31 c	0.32	6.52 a	0.38
1 ng	3.50 c	0.41	6.17 a	0.24
0.1 ng	2.94 a	1.00	5.70 a	1.18

[a] Growth promotion of canola seedlings treated with different amounts (100 μg to 0.1 ng per seed) of **1** measured as stem and root lengths (cm) 5 days after treatment. Values are means of 10 replicates. SD: ±standard deviation. Values with the same letter do not differ significantly ($P < 0.05$).

(Figure 3). Compound **1**, at 100 μg, completely arrested the in vitro growth of *R. solani*, while application rates lower than 10 μg produced only weak inhibition of the pathogen.

The effect of **1** on plant growth promotion was evaluated by measuring the length of canola (*Brassica napus*) seedlings whose seeds were previously coated with different amounts of the *Trichoderma* metabolite. Growth of canola seedlings was affected by treatments with **1** (Figure 4). This compound inhibited plant growth up to 45% and 33% (stem length) when applied at concentrations of 100 and 10 μg per seed, respectively. At concentrations of 100, 10, and 1 ng per seed, stem length increased by 42%, 44%, and 52%, respectively, compared to the control. However, 1 μg and 0.1 ng per seed did not significantly promote or inhibit the seedling growth (Table 2).

In a recent study we found evidence that secondary metabolites of *Trichoderma* spp. may have a role in both plant growth regulation

and activation of plant defense responses.[14] Our current results suggest a role of harzianic acid in plant growth regulation, as well as in the antagonism against fungal pathogens.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded with a Varian 400 instrument operating at 400 (^{1}H) and 125 (^{13}C) MHz, using residual and deuterated solvent peaks as reference standards. Mass spectra were obtained with a VG Autospec mass spectrometer. Flash chromatography was carried out using Merck silica gel 60 GF254, and precoated silica gel plates (Merck Kieselgel 60 GF_{254}, 0.25 mm) were used for TLC. The compounds were detected on TLC plates using UV radiation (254 or 366 nm) and/or by dipping the plates in a 10% (w/v) aqueous solution of $Ce(SO_4)_2$ in 2 M H_2SO_4 and heating at 110 °C for 10 min. The optical rotation was measured using a Perkin-Elmer 141 polarimeter and a microcell of one decimeter path length at 25 °C. Melting points were measured on a Reichert melting point apparatus, equipped with a Reichert microscope (objective 10/0.25 160/−) and are uncorrected.

Fungal Strains. All the fungal strains were maintained on potato dextrose agar (PDA) slants at room temperature and subcultured bimonthly. *R. solani*, *P. irregulare*, and *S. sclerotiorum* were isolated from field crops in Western Australia and cultured as above. *T. harzianum* was isolated from composted hardwood bark and was found to be suppressive to *P. irregulare*. This strain of *T. harzianum* has been deposited in the Soil Science and Plant Nutrition collection at the University of Western Australia, as M10.

Liquid Culture and Metabolite Production. Ten 7 mm diameter plugs of *T. harzianum*, obtained from actively growing margins of PDA cultures, were inoculated into 5 L conical flasks containing 1 L of sterile potato dextrose broth. The stationary cultures were incubated for 21 days at 25 °C. The cultures were filtered under vacuum through filter paper (Whatman No. 4).

Extraction and Isolation of *T. harzianum* Secondary Metabolites. The filtered culture broth (3 L) was acidified to pH 4 with 5 M HCl and extracted exhaustively with ethyl acetate. The combined organic fraction was dried (Na_2SO_4) and evaporated in vacuo at 35 °C. The red residue recovered was fractionated by flash column chromatography (silica gel; 200 g), eluting with a gradient of EtOAc/petroleum ether (1:1 to 10:0). Fractions showing similar TLC profiles were combined and further separated by preparative TLC (silica gel; $CHCl_3$/MeOH, 95:5).

Twelve fractions were collected, and of these, fraction 3 (592 mg) contained the bulk of material recovered. Fraction 3 was recrystallized from ethyl acetate at 4 °C to give 2-hydroxy-2-[4-(1-hydroxyocta-2,4-dienylidene)-1-methyl-3,5-dioxopyrrolidin-2-ylmethyl]-3-methylbutyric acid (**1-A** and **-B**) as yellow crystals: mp 85−86 °C; $[\alpha]_D$ +58.5 ($CHCl_3$; *c* 1.0); $[\alpha]_D$, as reported by Sawa et al.,[15] +19.6 (MeOH; *c* 1.06). UV, IR, ^{1}H NMR, ^{13}C NMR, and HR-FABMS were identical to those reported by Sawa et al.[15]

X-ray Structure Determination. The structure of **1** is shown in Figures 1 and 2, where the ellipsoids have been drawn at the 50% probability level. A full sphere of data was collected over several days to θ_{max} of 67.45° at 100(2) K on an Oxford Diffraction Gemini Ultra diffractometer fitted with Cu Kα radiation, yielding 20 616 reflections, these merging to 3396 unique after multiscan absorption correction (R_{int} = 0.0337), with 3349 reflections having $I > 2\sigma(I)$. The structure was refined against F^2 with full-matrix least-squares using the program SHELXL-97.[24] The Flack parameter refined to −0.04(11). Anisotropic displacement parameters were employed for the non-hydrogen atoms. Hydroxyl H-atoms were allowed to refine without restraints. All remaining H-atoms were added at calculated positions and refined by use of a riding model with isotropic displacement parameters based on the isotropic displacement parameter of the parent atom. Crystal data: $C_{19}H_{27}NO_6$, M = 365.42, monoclinic, space group $P2_1$, T = 100(2) K, a = 6.5570(2) Å, b = 20.0101(4) Å, c = 7.6041(2) Å, β = 105.323(3)°, V = 962.24(5) Å^3, Z = 2, ρ_{calc} = 1.261 g·cm^{-3}, μ = 0.774 mm^{-1}, $T_{max/min}$ = 1.00/0.86. Final R indices: R_1 = 0.0258 ($I > 2\sigma(I)$), wR_2 = 0.0662 (all data). Full details of the structure determination of **1** have been deposited with the Cambridge Crystallographic Data Centre as CCDC #745241. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk; or http://www.ccdc.cam.ac.uk.).

Antifungal Assays. Compound **1** was tested against the soil-borne pathogens *S. sclerotiorum*, *R. solani*, and *P. irregulare*. The method described by Dunlop et al.[25] was used, with some modifications. Pathogen plugs (5 mm diameter) from growing edges of colonies were placed at the center of Petri dishes containing 1/5 strength PDA. Samples of 10 μL of **1** at concentrations ranging from 0.01 to 200 μg plug^{-1} were applied on top of each plug. The controls were obtained by applying 10 μL of the solvent (ethyl acetate). The solvent was evaporated in a laminar flow cabinet, and the plates were incubated at 25 °C for six days. Growth of the pathogens was measured daily as colony diameter (mm). Each treatment consisted of four replicates, and the experiment was repeated twice.

Plant Growth Promotion Assay. To test the plant growth promotion activity of harzianic acid, canola (*Brassica napus* cv. Westar) seeds were coated with different amounts of **1** (100 μg to 0.1 ng per seed). Before the treatments, seeds were surface sterilized using 70% EtOH for 2 min, followed by 2% NaClO for 2 min, and then thoroughly washed with sterile distilled water.

Coated canola seeds were placed in the center of a water agar (1.5% w/v) Petri dish and allowed to germinate. Untreated seeds were used as controls. The seedlings were grown for 6 days in a controlled environment (25 ± 2 °C, 80% relative humidity, and a circadian cycle of 14 h of light and 10 h of dark), and the lengths of stems and roots were measured daily. Each treatment consisted of 10 replicates, and the experiments were repeated three times. Data from the experiments were combined since statistical analysis determined homogeneity of variance ($P \leq 0.05$).

Acknowledgment. F.V. gratefully acknowledges the support of Endeavour Research Fellowship Award by the Australian Government (Department of Education, Employment and Workplace Relations). Work by the authors F.V., R.M., and M.L. has been supported by the following projects: MIUR-PON number 10418; CNR project Endure Noex 2007−2010.

Supporting Information Available: X-ray data for harzianic acid are available free of charge via the Internet at http://pubs.acs.org.

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NP900548P