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Viridenepoxydiol, a New Pentasubstituted Oxiranyldecene Produced by *Trichoderma viride*

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A new pentasubstituted oxiranyldecene, named viridenepoxydiol, has been isolated (0.9 mg/L) from the culture filtrate of a strain of *Trichoderma viride* showing in vitro and in vivo antagonistic activity against *Sclerotium rolfsii*, which is the causal agent of crown and stem rot of artichoke. Viridenepoxydiol was characterized as 3,5,9-trimethyl-2-oxiranyl-dec-8-ene-2,5-diol (**3**) using spectroscopic methods. It showed inhibitor effect on mycelial growth of *S. rolfsii* and its minimum inhibitory concentration (over 90% inhibition) was found to be 396 μg/mL. This is the first time that viridenepoxydiol was reported.

KEYWORDS: ***Trichoderma viride*; oxiranyldecene; viridenepoxydiol; antifungal activity; *Sclerotium rolfsii*; biocontrol**

INTRODUCTION

As part of a long-running project searching for fungi suitable for biological control of soil-borne plant pathogens, we found a strain of *Trichoderma viride* that showed in vitro and in vivo antagonistic activity toward *Sclerotium rolfsii*, the causal agent of crown and stem rot artichoke, which has a great economic importance on artichoke in Sardinia (*1, 2*). *Trichoderma* strains produce a considerable number of metabolites that exhibit antibiotic activity and are probably involved in the biocontrol process (*3, 4*). This strain of *T. viride* has previously been shown to produce bioactive metabolites such as isoharziandione (*5*), 6-pentyl-α-pyrone, and viridepyronone (*6*) able to inhibit mycelial growth of *S. rolfsii*. In continuation of this work, we have examined in more detail the other metabolites produced by this strain.

This paper reports the chemical and biological characterization of a new metabolite, named viridenepoxydiol, produced in liquid cultures by the above *T. viride* strain. This is the first time that viridenepoxydiol has been reported.

MATERIALS AND METHODS

Fungal Strains, Culture Medium, and Growth Conditions. *Sclerotium rolfsii* was originally isolated from infected artichoke plants (*Cynara scolymus*) in Sardinia (Italy) and was maintained on potato dextrose agar (PDA) in 9-cm-diameter Petri dishes under ambient conditions. *Trichoderma viride* was isolated from forest soil collected in Sardinia and deposited at the collection of the Dipartimento di Protezione delle Piante, Università degli Studi di Sassari, Italy, as IPVS 1817. Slant cultures on PDA of the fungus were stored in a refrigerator at 4 °C. This strain was grown on a Czapek medium under conditions as previously reported (*6*).

General Experimental Procedures. Optical rotations were measured in $CHCl_3$ solution on a Jasco (Tokyo, Japan) P-1010 digital polarimeter; elemental analyses were perfomed on a Fison (Manchester, U.K.) instrument EA 1108 Elemental Analyzer; infrared (IR) spectra were recorded neat on a Perkin-Elmer (Norwalk, CT) Spectrum One FT-IR spectrometer and UV spectra were taken in CH_3CN solution on a Perkin-Elmer (Norwalk, CT) Lambda 25 UV/Vis spectrophotometer; ^1H and ^{13}C NMR spectra were recorded at 400 or 600 MHz and at 150 or 75 MHz, respectively, in $CDCl_3$, on Bruker (Kalsruhe, Germany) spectrometers of the Istituo di Chimica Biomolecolare del CNR, Pozzuoli, Italy. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectrum (*7*). DEPT, COSY-45, HSQC, HMBC, and NOESY experiments (*7*) were performed using standard Bruker microprograms. An electrospray MS (ESI) spectrum was recorded on a Perkin-Elmer (Norwalk, CT) API 100 LC-MS; a probe voltage of 5300 V and a declustering potential of 50 V were used. A HR ESI MS spectrum was recorded on Micromass Q-Tof Micro (Waters, Milford, MA). Analytical and preparative TLC were performed on Kieselgel 60, F_{254}, 0.25 and 0.5 mm, respectively (Merck, Darmstadt, Germany) or on Stratocrom KC-18, F_{254}, 0.25 mm (Whatman, Clifton, NY) reversed phase plates; spots were visualized with UV (254 or 366 nm) and/or spraying with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography was performed on Kieselgel 60, 0.063−0.20 mm (Merck).

Extraction and Purification of *T. viride* Metabolites. The culture filtrates (10 L) were acidified to pH 5.0 with 2 N HCl and extracted

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of Viridenepoxydiol (**3**)[a,b]

C	δ[c]	^{1}Hδ	J (Hz)	HMBC
1	26.0 (q)	1.26 (s)		1.86, 1.62
2	81.3 (s)			1.88, 1.86, 1.69, 1.56, 1.26, 1.07
3	44.2 (d)	1.62 (m)		1.86, 1.56, 1.26, 1.07
4	24.5 (t)	1.86 (m)		1.62, 1.49,1.17
		1.56 (m)		
5	74.9 (s)			2.05, 1.86, 1.62, 1.56, 1.17
6	40.4 (t)	2.05 (m)		5.14, 2.05, 1.86, 1.56, 1.17
		1.49 (t)	8.3, 7.0	
7	22.7 (t)	2.05 (m)		1.49
8	124.5 (d)	5.14 (br t)	7.0, 1.6	2.05, 1.69, 1.62, 1.49
9	131.8 (s)			2.05, 1.69, 1.62
10[d]	25.7 (q)	1.69 (br s)		5.14, 1.62
1′	54.3 (d)	1.86 (m)		1.88, 1.69, 1.62, 1.07
2′	40.3 (t)	1.88 (m)		1.86, 1.62, 1.26
		1.69 (m)		
3′	14.5 (q)	1.07 (d)	(6.8)	1.86, 1.62
4′	25.0 (q)	1.17 (s)		
5′[d]	17.7 (q)	1.62 (br s)		5.14, 1.69
OH	1.47 br s			

[a] The chemical shifts are in δ values (ppm) from TMS. [b] 2D ^{1}H,^{1}H (COSY) and 2D ^{13}C,^{1}H (HSQC) NMR experiments delineated the correlations of all protons and the corresponding carbons. [c] Multiplicities determined by DEPT spectrum. [d] These assignments could be exchanged.

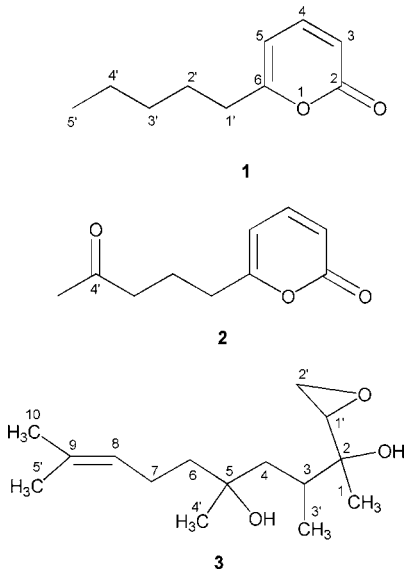

Figure 1. Structures of 6-*n*-pentyl-2*H*-pyran-2-one (**1**), viridepyronone (**2**), and viridenepoxydiol (**3**), the antifungal pentasubstituted oxiranyldecene produced by *Trichoderma viride*.

exhaustively with ethyl acetate. The organic extracts were combined, dried with Na_2SO_4, and evaporated under reduced pressure at 40 °C to give a red brown oily residue (1.4 g). This residue was highly active in inhibiting the mycelial growth of *S. rolfsii*. The ethyl acetate extract was then submitted to bioassay-guided fractionation by silica gel column chromatography eluted with a gradient of petroleum ether−ethyl acetate (20:1 → 0:100) and ethyl acetate−methanol (10:1 → 0:100). The collected fractions (15 mL each) were combined into 12 groups (T_1−T_{12}) on the basis of their silica gel TLC profiles. All fractions were assayed for their antifungal activity against *S. rolfsii*. The fractions T_4, T_5, and T_7 were found the most active against *S. rolfsii*.

Purification of fractions T_4 (70 mg), T_5 (130 mg), and T_7 (65 mg) by preparative silica gel TLC afforded three known compounds: isoharziandione (6 mg/L), 6-pentyl-α-pyrone (**1**, 11 mg/L), and viridepyronone (**2**, 0.8 mg/L), respectively (*5*, *6*). The fraction T_7 contained another metabolite (20 mg), showing moderate activity against *S. rolfsii*. The latter was purified by two successive steps of preparative silica gel TLC and analytical reversed-phase TLC eluted by $CHCl_3$-*i*-PrOH (30:1) and MeCN−H_2O (60:40), yielding 9 mg of viridenepoxydiol (**3,** 0.9 mg/L), as a homogeneous oil resistant to crystallization [R_f 0.58 and 0.31, by silica gel and reversed-phase TLC, eluent systems $CHCl_3$−MeOH (95:1) and MeCN−H_2O (60:40), respectively].

Spectroscopic Data of Viridenepoxydiol (3). Colorless oil. $[\alpha]^{25}_D$ −5.0 (*c* 0.2). Found: C, 70.57; H, 11.21; O, 18.92. $C_{15}H_{28}O_3$ requirement: C, 70.27; H, 11.01; O, 18.72. UV λ_{max} (log ϵ): 192 (3.96) nm. IR ν_{max} 3436, 1454, 1376 cm^{-1}. ^{1}H and ^{13}C NMR, see **Table 1**. HR ESI-MS *m*/*z* 295 $[M + K]^+$, 279.1924 $[M + Na]^+$ (calcd for $C_{15}H_{28}O_3Na$, 279.1936), 263 $[M + K - MeOH]^+$, 257 $[M + H]^+$, 239 $[M + H - H_2O]^+$, 222 $[M + H - H_2O - OH]^+$, 205 $[M + H - H_2O - 2 \times OH]^+$.

Fungitoxicity Tests. Crude extracts, chromatographic fractions, and pure compound were tested for antifungal activity against *S. rolfsii*. PDA plates (90 mm) were centrally inoculated with a 6-mm-diameter agar plug of fungus, taken from the growing margin of a 3-day colony on PDA. The assay was performed by applying 20 μL of the chromatographic fractions and viridenepoxydiol solutions (25 and 5 μg/μL, respectively) directly to the inoculum surface. All the bioassays included controls with solvent. Inhibition percentage was evaluated 3 days after treatment at 25 °C. Each experiment was repeated twice and contained three replicates each time. Minimum inhibitory concentration (over 90% inhibition) of viridenepoxydiol was determined. For this purpose, the compound (20 mg) was dissolved in acetone (285 μL), serially diluted in the same solvent, and added to PDA at 48 °C. Four milliliters of the medium was added in a 6-cm-diameter Petri dish. The final concentrations ranged from 50 to 350 μg/mL. The experiment was carried out as previously reported (*6*).

Data Analysis. Bioassay experiments on antifungal effects and minimum inhibitory concentration of viridenepoxydiol were analyzed by STATGRAPHICS PLUS software. Means for each experiment were compared by using Duncan's multiple range test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Extraction and Purification of Viridenepoxydiol. The culture filtrate of *T. viride* was extracted exhaustively with ethyl acetate, yielding a brown oily residue (1.4 g) that completely inhibited the mycelial growth of *S. rolfsii*. The crude extract was fractionated by using a combination of column chromatography and TLC. The obtained fractions (T_1−T_{12}) were tested for their antifungal activity against *S. rolfsii*. The fractions T_4, T_5, and T_7 caused total inhibition of mycelial growth of *S. rolfsii*. The residue left from group T_4 was further purified by TLC to yield isoharziandione (*5*) while *6*-*n*-pentyl-2*H*-pyran-2-one (**1**, **Figure 1**) and viridepyronone (**2**, **Figure 1**) were isolated from the fraction groups T_5 and T_7, respectively (*6*). Moreover, fraction T_7 contained another metabolite (20 mg), showing antifungal activity, that was further purified by two successive TLC steps, yielding 9 mg (0.9 mg/L) of a new metabolite, named viridenepoxydiol (**3**, **Figure 1**), as a homogeneous oil resistant to crystallization.

Structural Elucidation of Viridenepoxydiol 3. Compound **3** had a molecular weight of 256, corresponding to a molecular formula of $C_{15}H_{28}O_3$, consistent with two unsaturations, as deduced from the values of 279.1924 recorded for the peak of its sodium cluster $[M + Na]^+$ in the HR ESI mass spectrum and from its elemental analysis. Absorption bands typical of hydroxy, olefinic, and methyl groups were observed in the IR spectrum (*8*), while the UV spectrum showed the typical absorption of a trisubstituted olefinic group (*9*).

In fact, a preliminary ^{1}H NMR spectrum (**Table 1**) showed a broad signal of hydroxy groups at δ 1.47, that of an olefinic proton appearing as a broad triplet at δ 5.14 (H-8), and those of five methyl groups and some complex systems in the aliphatic region of δ 2.1−1.0 (*9*). Two of these methyl groups at δ 1.69

Table 2. 2D ^{1}H-NOE (NOESY) Spectroscopic Data Obtained for Viridenepoxydiol (**3**)

considered	effects	considered	effects
5.14 (H-8)	2.05 (H_2-7 and/or H-6A), 1.69 (Me-10), 1.49 (H-6B)	1.62 (H-3)	1.26 (Me-1), 1.07 (Me-3′)
2.05 (H_2-7 and/or H-6A)	5.14 (H-8), 1.49 (H-6B), 1.17 (Me-4′)	1.56 (H-4B)	1.17 (Me-4′), 1.07 (Me-3′)
1.88 (H-2′A)	1.26 (Me-1), 1.07 (Me-3′)	1.49 (H-6B)	5.14 (H-8), 2.05 (H_2-7 and/or H-6A), 1.17 (Me-4′)
1.86 (H-4A and/or H-1′)	1.17 (Me-4′), 1.26 (Me-1) 1.07 (Me-3′)	1.26 (Me-1)	1.88 (H-2′), 1.86 (H-1′), 1.62 (H-3), 1.07 (Me-3′)
1.69 (Me-10)	5.14 (H-8), 1.62 (Me-5′)	1.17 (Me-4′)	2.05 (H_2-7 and/or H-6A), 1.86 (H-4A), 1.56 (H-4B), 1.49 (H-6B)
1.69 (H-2′)	1.07 (Me-3′)	1.07 (Me-3′)	1.88 (H-2′A), 186 (H-4A and/or H-1′), 1.69 (H-2′B), 1.62 (H-3) 1.56 (H-4B), 1.26 (Me-1)
1.62 (Me-5′)	1.69 (Me-10)		

and 1.62 (Me-10 and Me-5′) being long-range coupled into the COSY spectrum (*7*) with the olefinic proton (H-8) were bonded to the double bond. H-8 also coupled in the same spectrum with the proton of the adjacent methylene group, appearing as a complex system at δ 2.05 (H_2C-7), while the proton signal of another methylene (H_2C-6) overlapped those of H_2C-7 and correlated with the geminal one present as a triplet (J = 8.3 and 7.0 Hz) at δ 1.49. The other two methyl groups appearing as singlets at δ 1.26 and 1.17 (Me-1 and Me-4′) were probably linked to two hydroxylated quaternary carbons (C-2 and C-5, respectively). The remaining methyl group, resonating as a doublet (J = 6.8 Hz) at δ 1.07 (Me-3′), was coupled in the COSY spectrum with a proton (H-3) appearing as a multiplet at δ 1.62 in part overlapped from the signal of Me-5′. This latter proton (H-3) coupled in the same spectrum with both the protons of another methylene group (H_2C-4) resonating both as multiplets at δ 1.86 and 1.56, respectively. Finally, the COSY spectrum also showed the presence of another methylene group (H_2C-2′) whose protons resonating as multiplets at δ 1.88 and 1.69 were coupled to each other and to a multiplet at δ 1.86 attributed to a proton (H-1′) bonded to a secondary carbon. The latter, considering also the typical chemical shift of the corresponding carbon (*9*, *10*), probably belonged to a monosubstituted oxiran ring, which was the end tail of the compound. The couplings observed in the COSY spectrum were confirmed by the results of a series of double-resonance decoupling experiments. In particular, these spectra confirmed the couplings between the protons of the methine (CH-1′) and methylene (CH_2-2′) groups of the oxiran ring as well as those between H-3 with both CH_3-3′ and CH_2-4, and finally between the olefinic proton (H-8) and the protons of the adjacent methylene group (CH_2-7) and between the latter and the protons of the CH_2-6. Obviously, the same experiment confirmed that all methylene protons are themselves coupled. These structural features were confirmed by the analysis of the ^{13}C NMR spectrum (**Table 1**) of **3** which showed the presence of quaternary and secondary olefinic carbons at δ 131.8 and 124.5 (C-9 and C-8) that of the two vinyl methyl groups at δ 25.7 and 17.7 (Me-10 and Me-5′), while the two methyl groups bonded to quaternary oxygenated carbons and the secondary methyl group resonated at δ 26.0, 25.0, and 14.5 (Me-1, Me-4′, and Me-3′) (*9*, *10*). The four methylene and the two methine groups, assigned on the basis of the correlation observed in the HSQC spectrum (*9*), were present at δ 40.4, 40.3, 24.5, and 22.7 (C-6, C-2′, C-4, and C-7) and at δ 54.3 and 44.2 (C-1′ and C-3). The correlations observed in the HSQC spectrum further confirmed the assigments of the proton spectrum. Finally, the two quaternary hydroxylated carbons resonated at δ 81.3 and 74.9 (C-2 and C-5) (*9*, *10*). On these bases, all the protons and the corresponding carbons chemical shifts were assigned (**Table 1**), and viridenepoxydiol was formulated as 3,5,9-trimethyl-2-oxiranyl-dec-8-ene-2,5-diol (**3**).

The structure of **3** was supported by the ^{1}H,^{13}C long-range correlations observed in the HMBC spectrum (**Table 1**) (*7*) and by MS spectra. In particular, the HMBC spectrum showed the significant couplings between the protons of the Me-1, Me-3′, and Me-4′ with the carbons C-2, C-3, and C-2′, C-1, C-2, and C-1′, and C-4, C-5, and C-6, respectively. The HR ESI-MS spectrum, in addition to the sodium cluster $[M + Na]^+$ at *m*/*z* 279.1924, also showed that formed with the potassium $[M + K]^+$ and the pseudomolecular $[M + H]^+$ ions at *m*/*z* 295 and 257, respectively. Some typical fragmentation peaks, which were generated from the potassium clustered ion $[M + K]^+$ by a loss of MeOH, or alternatively from the pseudomolecular ion $[M + H]^+$ by successive losses of H_2O and two OH residues, were observed at *m*/*z* 263 and 239, 222, and 205, respectively (*9*).

Knowing the absolute configuration of natural products has become crucial because it provides essential information for both total synthesis and molecular mode action of bioactive metabolites. The absolute configuration of natural compounds have, in some cases, been determined by X-ray analysis or synthetic work. Unfortunately, viridenepoxydiol is an oil resistant to crystallization and is laborious to synthesize because of its numerous chiral centers. NOE-based methods in combination with molecular mechanics calculations have been proposed for configuration assignment of flexible molecules, particularly for macrocyclic compounds, as macrolides and other compounds. However, even with new NOE-based techniques it is still very difficult to assign the stereochemical configuration of highly flexible carbon chains because the presence of multiple conformers, in which minor populations often make disproportionately large contributions to NOE intensity, occasionally leads to contradictory distance constraints. This is the case of viridenepoxydiol for which the NOESY effects (**Table 2**) recorded between the protons of different moieties can only strongly support the structure assigned to **3**. In fact, particularly significant are the effects observed between H-1′ and Me-1 and between the latter and Me-3′. Furthermore, the two new methods developed for the stereochemical determination of acyclic and small organic compounds, respectively, based on carbon−proton spin-coupling constants (*11*) and residual dipolar couplings (*12*) were inapplicable for **3**. In fact, the first method requires the presence of stereogenic adjacent methine carbons substituted with a methyl or a hydroxy (alkoxy) group in the analyzed compound, while the latter one is still restricted only to water-soluble compounds. Unfortunately, viridenepoxydiol contains two stereogenic suitable functionalized methines (C-3 and C-1′) but alternate with two chiral quaternary carbons, and it is not soluble in water.

Some decene derivatives are reported as components of spice flavors (*13*) and as metabolites of insects (*14*) and fungi (*15*), while the oxiran group is present as a structural feature important for the activity in several classes of bioactive fungal metabolites such as sesquiterpene eremophilanes (*16*), trichothecenes, verrucarins, and cytochalasins (*17*−*19*), and sphaeropsidones (*20*). The oxiranyldecenes have only been reported as synthetic compounds (*21*). Therefore, viridenepoxydiol is the first example of naturally occurring oxiranyldecene. To our knowledge,

cyclonerodiol, a sesquiterpenoid produced by different fungi, including *Giberella*, *Fusarium*, *Trichoderma*, and *Trichothecium* species but lacking antifungal activity (*22*), is the microbial metabolite closest to viridenepoxydiol.

Fungitoxicity Tests. Antifungal bioassay indicated that viridenepoxydiol was effective in inhibiting the growth of *S. rolfsii* by 100% at the concentration tested (5 μg/mL). The results of the MIC test showed that the concentration of viridenepoxydiol was correlated ($R^2 = 0.98$) with the inhibition percentage of mycelial growth of *S. rolfsii* in PDA plates. The relationship equation between the concentration of viridenepoxydiol (x) and inhibition percentage of mycelial growth of *S. rolfsii* (y) was $y = 40.51 - 0.35x + 0.0012x^2$. The minimum concentration of viridenepoxydiol for inhibition of *S. rolfsii* was 396 μg/mL.

The isolation of viridenepoxydiol provides new information on the production in vitro of antibiotic metabolites by *Trichoderma viride* commonly employed for developing suitable strategies of biological control against several plant pathogens such as *Colletotrichum capsici*, *Sclerotinia sclerotiorum*, *Pythium aphanidermatum*, *Fusarium oxysporum* f. sp. *lycopersici*, *Alternaria brassicola*, *A. alternata*, *Phomopsis vexans*, *Macrophomina phaseolina*, and *Rhizoctonia solani* isolated from chili (*Capsicum* spp.), cauliflower (*Brassica oleracea*), tomato (*Lycopersicon esculentum*), and eggplant (*Solanum melongena*) plants (*23*).

T. viride synthesizes bioactive metabolites belonging to different classes of natural compounds such as the 2*H*-pyran-2-ones, tetracyclic diterpenes, and oxiranyldecenes. The biological activities of secondary metabolites containing the 2*H*-pyran-2-one moiety are well-documented, and they have been reported to be produced by fungi belonging to several genera (*24*−*28*). The oxiran group also plays an important role in the biological activity of different natural compounds (*16*−*20*), so its presence could be essential to the antifungal activity of viridenepoxydiol.

Future investigations should be addressed to clarify the role of bioactive metabolites produced by this strain of *T. viride* in the biocontrol process, and the nature of interactions between them or with enzymes.

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