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Viridepyronone, a New Antifungal 6-Substituted 2*H*-Pyran-2-one Produced by *Trichoderma viride*

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A new antifungal 6-substituted 2*H*-pyran-2-one, named viridepyronone, has been isolated from a cultural filtrate of a strain of *Trichoderma viride* showing antagonistic activity *in vitro* toward *Sclerotium rolfsii*, which is the causal agent of crown and stem rot of artichoke. Viridepyronone was characterized as 6-(4-oxopentyl)-2*H*-pyran-2-one **2** with spectroscopic methods. Bioassays showed that viridepyronone had a good antifungal activity against *S. rolfsii*, and its minimum inhibitory concentration (over 90% inhibition) was found to be 196 μg/mL. This is the first report of viridepyronone produced by any species of fungi.

KEYWORDS: ***Trichoderma viride*; pyran-2-one; viridepyronone; antifungal activity; *Sclerotium rolfsii*; crown and stem rot; biocontrol**

INTRODUCTION

Seeking fungi suitable for the biological control of soil-borne plant pathogens, a strain of *Trichoderma viride* was found that showed antagonistic activity, *in vitro* and *in vivo*, toward *Sclerotium rolfsii*, the causal agent of crown and stem rot of artichoke (*1*, *2*). The antagonistic activity exhibited *Trichoderma* spp. strains may in part be explained by the production of different classes of bioactive metabolites, including antibiotics, which are inhibitors of fungal growth and enzymes (*3*−*5*).

We have previously isolated and characterized isoharziandione, a new tetracyclic diterpene, from the culture filtrates of strain IPVS 1817 of *T. viride* able to inhibit fungal growth of *S. rolfsii* (*6*). Here we report on a new metabolite produced in liquid cultures by the above *T. viride* strain and on the isolation and chemical characterization of viridepyronone, structurally related to 6-*n*-pentyl-2*H*-pyran-2-one. This paper is the first report on viridepyronone produced by fungi.

MATERIALS AND METHODS

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

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Fermentation. Conidial suspensions (2 mL) of *T. viride* were inoculated into 50 Roux flasks each containing 150 mL of Czapek medium fortified with 5% yeast extract (pH 5.9). The stationary cultures were incubated for 21 days at 25 °C in the dark. The cultures were filtered under vacuum through filter paper (Whatman N*o*. 1), and the filtrates stored at −20 °C until used for chemical analysis.

Extraction and Purification of Antifungal Metabolites. The combined culture filtrate (5 L) was concentrated under reduced pressure to approximately one-quarter of its original volume, acidified to pH 5.0 with 2 N HCl, and extracted exhaustively with ethyl acetate. The combined organic layers were dried over Na_2SO_4 and evaporated under reduced pressure at 30 °C to give a red-brown oily residue (900 mg). The antifungal activity of the extract was determined at a concentration of 10 mg/mL against *S. rolfsii* by its direct (40 μL) application to the paper disk surface. The ethyl acetate extract was found to be active against *S. rolfsii*, and was then submitted to bioassay-guided fractionation through column (80 × 3 cm) chromatography on silica gel (100 g), eluted with a gradient of $CHCl_3$−*i*-PrOH (100:1 → 1:2). The collected fractions (15 mL each) were monitored by TLC analysis and the resulting homogeneous fractions were combined into 10 groups, T_1−T_{10}. All the fractions were screened for their antifungal activity against *S. rolfsii* as described below. The fractions T_4, T_5, and T_7 were found the most active against *S. rolfsii*.

Purification of fractions T_4 (50 mg) and T_5 (120 mg) by a combination of column chromatography and preparative silica gel TLC gave two known compounds, isoharziandione (6 mg/L) and 6-pentyl-α-pyrone (**1**, 11 mg/L), respectively, which showed chromatographic and specrtoscopic properties identical with those of standard samples (*6*, *8*). The residue (30 mg) left from fraction T_7 was purified by two successive steps of preparative silica gel TLC eluted by $CHCl_3$−*i*-PrOH (20:1 and 95:5 v/v, respectively) and yielded 4 mg of viridepyronone (**2**, 0.8 mg/L) as a homogeneous oil resistant to crystallization [R_f 0.28, 0.31, and 0.68, by silica gel and reversed-phase TLC, eluent systems $CHCl_3$−*i*-PrOH (95:5), EtOAc−*n*-hexane (6:4), and EtOH−H_2O (1:1), respectively].

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General Experimental Procedures. Optical rotation was measured in $CHCl_3$ solution on a JASCO (Tokyo, Japan) DIP-370 digital polarimeter; IR and UV spectra were determined neat and in MeCN solution, respectively, on a Bio-Rad (Hercules, CA) Win FT-IR spectrometer and a Shimadzu (Kyoto, Japan) UV-1601 UV−visible spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded at 400 or 300 MHz and at 100 or 75 MHz, respectively, in $CDCl_3$, on Bruker (Kalsruhe, Germany) spectrometers. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra (*7*). DEPT, COSY-45, HSQC, and HMBC experiments (*7*) were performed by using standard Bruker microprograms. EI- and HR EI-MS (70 eV) employed a Fisons (Hastings, U.K.) Trio-2000 and a Fisons ProSpec spectrometer, respectively. Electrospray MS were 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. Analytical and preparative TLC were performed on silica gel (Kieselgel 60, F254, 0.25 and 0.5 mm, respectively, Merck, Darmstadt, Germany), or on reversed-phase (KC-18F Silica Gel A, 0.20 mm, Whatman, Clifton, NY) plates; spots were visualized with UV radiation (254 or 366 nm) and/or by dipping the plates in a 10% (w/v) aqueous solution of $KMnO_4$ or by spraying with 10% H_2SO_4 in MeOH followed by 5% phosphomolybdic acid in MeOH, and heating at 110 °C for 10 min. Column chromatography used silica gel (Kiesegel 60, 0.063−0.20 mm, Merck).

Spectroscopic Data for Viridepyronone (2). Colorless oil; $[\alpha]^{25}_D$ +4.4° (*c* 0.19); UV λ_{max} (log ϵ) 301 (3.66), 220 (3.49) nm; IR ν_{max} 1734, 1635, 1558 cm^{-1}; ^{1}H and ^{13}C NMR, see **Table 1**; HR EI-MS *m/z* (rel intensity) 181 $[M + H]^+$ (17), 180.0776 $[M]^+$ (calcd for $C_{10}H_{12}O_3$, 180.0787) (78), 165 $[M - Me]^+$ (3), 137 $[M - Me - CO]^+$ (27), 123 $[M + H - Me_2CO]^+$ (100), 95 $[M - C_5H_9O]^+$ (75), 58 $[CH_2{=}C(OH)CH_3]^+$ (44); ES-MS (+) *m/z* 219 $[M + K]^+$, 203 $[M + Na]^+$, 181 $[M + H]^+$, 123 $[M + H - Me_2CO]^+$.

Antifungal Activity. An agar diffusion method was utilized to test the antifungal activity of the *T. viride* fungal culture filtrate, its organic crude extract, the chromatographic fractions, and viridepyronone against *S. rolfsii*. To a 6-cm-diameter paper disk was applied culture filtrate (40 μL), crude extract (40 μL), chromatohraphic fraction (40 μL), and purified compound (0.5 mg/40 μL, corresponding to 6.9×10^{-2} M). The air-dried disks were placed on a PDA plate 2.0 cm from the plate edge. Each plate was then inoculated at the center with a 6-mm agar plug of *S. rolfsii*, removed from the margin of a 2-day-old colony on PDA. Inhibition percentage was evaluated 3 days after treatment at 25 °C from the equation as follows: $100(y - x)/y$, where y = growth diameter in untreated control and x = growth diameter in treatment. Each treatment consisted of three replicates. The experiment was repeated twice.

Minimum Inhibitory Concentration. Pure viridepyronone was added to PDA in a plate culture to determine its minimum inhibitory concentration (over 90% inhibition) against *S. rolfsii*. Viridepyronone (30 mg) was dissolved in methanol (also used as control), serially diluted in the same solvent, and aliquots added to the PDA at 48 °C. Five milliliters of the medium was added to a 6-cm-diameter Petri dish. The final concentrations were in the range 10−250 μg/mL. A 6-mm-diameter plug of *S. rolfsii*, removed from the margin of a 3-day-old colony on PDA, was placed in the center of the plate. The growth of *S. rolfsii* at 25 °C was recorded after 3 days of incubation. Each treatment consisted of three replicates. Inhibition percentage was obtained from the equation as described above. The experiment was repeated twice.

Data Analysis. Bioassay experiments on antifungal effects and minimum concentration of viridepyronone were analized 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 Viridepyronone. The crude oily residue (900 mg) obtained from the organic extraction (EtOAc) of *T. viride* culture filtrates was fractionated by using a combination of column chromatography and TLC steps, and 10 pooled fractions, T_1−T_{10}, were obtained. All the fractions were tested for their antifungal activity against *S. rolfsii*. Fractions T_4, T_5, and T_7 caused marked inhibition of mycelial growth of *S. rolfsii* at a concentration of 0.8 mg/40 μL. The residue (50 mg) left from group T_4 was further purified by TLC yielding isoharziandione (*6*). The residue (120 mg) left from fraction group T_5 of the original column gave *6-n*-pentyl-2*H*-pyran-2-one (**1**, **Figure 1**) (*8*) as a colorless liquid with a strong coconut-like odor. The residue (30 mg) left from fraction group T_7 was further purified with two successive steps of preparative TLC yielding 4 mg (0.8 mg/L) of a new antifungal metabolite named viridepyronone (**2**, **Figure 1**) as a homogeneous oil resistant to crystallization.

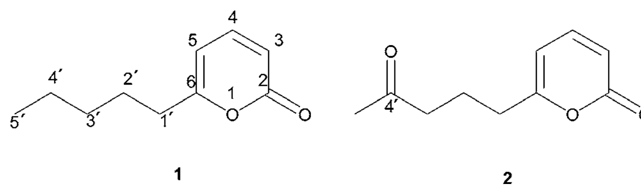

Figure 1. Structure of 6-*n*-pentyl-2*H*-pyran-2-one (**1**) and viridepyronone (**2**), the antifungal α-pyrones produced by *Trichoderma viride*.

Table 1. ^{1}H and ^{13}C NMR Spectral Data for Viridepyronone (**1**)[a,b]

C	δ^c	^{1}H	*J*, Hz	HMBC
2	162.4 (s)			7.25, 6.16
3	113.5 (d)	6.16 (d)	9.4	5.98
4	143.05 (d)	7.25 (dd)	9.4, 6.5	
5	102.9 (d)	5.98 (d)	6.5	2.51
6	165.6 (s)			7.25, 5.98, 2.51,1.95
1′	32.8 (t)	2.51 (t)	7.3	5.98, 2.51, 1.95
2′	20.8 (t)	1.95 (tt)	7.3, 7.3	2.51
3′	42.1 (t)	2.51 (t)	7.3	2.51, 2.15, 1.95
4′	207.6 (s)			2.51, 2.15, 1.95
5′	29.4 (q)	2.15 (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 the DEPT spectrum.

Structure Determination of Viridepyronone 2. Mass spectroscopy indicated a molecular ion $[M]^+$ for compound **2** of *m/z* 180, corresponding to a molecular formula of $C_{10}H_{12}O_3$. Absorption bands typical of saturated and α,β-unsaturated carbonyl groups were observed in the IR spectrum, while the UV spectrum showed the absorption maximum typical of an α-pyrone.

In fact, preliminary ^{1}H and ^{13}C NMR investigation showed signals very similar to those described for **1**. ^{1}H NMR (**Table 1**) and COSY (*7*) spectra showed a doublet of doublets and two doublets at δ 7.25, 6.16, and 5.98, which are in agreement to those of **1** (*8*) and were attributed to the protons H-4, H-3, and H-5 of an α-pyrone ring (*9*). The presence of this moiety in **2** was also supported by the chemical shifts recorded in the HSQC (*7*) spectrum for the corresponding carbons at δ 143.5, 113.5, and 102.9 (C-4, C-3, and C-5, respectively), as well as for the signals of the ester carbonyl (C-2) and quaternary (C-6) carbons observed in the ^{13}C NMR spectrum (**Table 1**) at δ 162.4 and 165.6, both typical chemical shifts for 6-substituted α-pyrone (*8*, *10*).

Considering that **2** differed from **1** only by an extra oxygen and two fewer hydrogen atoms, these differences are certainly located in the side chain attached to C-6, which in **1** is a *n*-pentyl while in **2** it appeared to be a 4-oxopentyl. In fact, the ^{1}H NMR spectrum of **2** showed the presence of a singlet and a triplet (J = 7.3 Hz) at δ 2.15 and 2.51, typical of a methyl (CH_3-5′) and a methylene (CH_2-3′) adjacent to a carbonyl group (O=C-4′)

(*9*), which in the ^{13}C NMR spectrum appeared at the typical chemical shift value of δ 207.6 (*10*). The methylene group (CH_2-3′) in the COSY spectrum coupled with a multiplet (J = 7.3 Hz) at δ 1.95 due to another methylene group (CH_2-2′), which appeared as a quintet due to a further coupling with another adjacent methylene group (CH_2-1′). The latter, resonating as a triplet (J = 7.3 Hz), perfectly overlapped with that of CH_2-3′ at δ 2.51, and proved to be attached to the α-pyrone ring by the long-range coupling observed between C-1′ and H-5 in the HMBC (*7*) spectrum. In the HSQC spectrum, the three methylene and the methyl groups coupled with carbons appearing at the expected chemical shift values of δ 42.1, 32.8, 20.8, and 29.4 for C-3′, C-1′, C-2′, and C-5′ respectively (*10*).

All the protons and the corresponding carbon chemical shifts were assigned (**Table 1**) on this basis, and viridepyronone was identified as 6-(4-oxopentyl)-2*H*-pyran-2-one (**2**).

The structure of **2** was supported by the $^{1}H,^{13}C$ long-range correlations observed in the HMBC spectrum (**Table 1**) (*7*), and by MS spectra. The HR EI-MS spectrum, in addition to the molecular ion at 180.0776, showed ions generated by fragmentation mechanisms typical of α-pyrone and saturated ketone containing compounds (*9*, *11*). Loss of the side chain, which is a fragmentation typical of 6-substituted α-pyrone, generated the characteristic ion at *m/z* 95 (*11*), while the successive loss of Me and CO generated the ions at *m/z* 165 and 137 (*9*). Finally, the ion $[CH_2{=}C(OH)CH_3]^+$, generated by the expected McLafferty rearrangement, was observed at *m/z* 58 (*9*, *11*). The pseudomolecular ion $[M + H]^+$ at *m/z* 181, frequently observed for lactone-ring containing compounds (*9*), also generated the base peak ion recorded at *m/z* 123 via loss of acetone (*9*). This latter ion was also observed in the ES-MS spectrum together with the potassium $[M + K]^+$ and the sodium $[M + Na]^+$ clusters and the pseudomolecular ions at *m/z* 219, 203, and 181, respectively.

Pyran-2-ones (α-pyrones) are a group of naturally occurring compounds which are broadly distributed in nature as plant, animal, marine organism, and microbial metabolites, often with interesting biological activity (*12*−*14*), and the total synthesis of some of them has been achieved (*14*). Other secondary metabolites containing the pyran-2-one moiety are produced by fungi belonging to several genera including *Alternaria*, *Aspergillus*, *Fusarium*, and *Trichoderma*, and exhibit antibiotic, antifungal, cytotoxic, neurotoxic, and phytotoxic activities (*15*). α-Pyrones structurally related to **2** have been reported as products obtained by microbial transformation of **1** by *Botrytis cinerea* (*8*), *Sclerotinia sclerotiorum*, *Fusarium crookwellens*, and a number of *Penicillium* isolates (*16*) and as the bioactive 6-substituted-5,6-dihydropyran-2-ones, which were isolated from aerial parts of *Piper reticulatum* (*17*).

Antifungal Activity. The paper disk assay showed that viridepyronone was effective in inhibiting the growth of *S. rolfsii* by 48%. The culture filtrate of strain IPVS-1817 of *T. viride* and its crude organic extract showed an inhibition of 19% and 28%, respectively.

MIC of Viridepyronone against *S. rolfsii*. The concentration of viridepyronone was found to be correlated ($R^2 = 0.97$) to the inhibition of mycelial growth of *S. rolfsii* in the PDA plates assay. The relationship equation between concentration of viridepyronone (x) and inhibition percentage of mycelial growth of *S. rolfsii* (y) was $y = -8.99 + 0.74x - 0.0012x^2$. The minimum concentration of viridepyronone for inhibition of *S. rolfsii* was 196 μg/mL. The mycelial growth of *S. rolfsii* was completely inhibited by viridepyronone at 246 μg/mL.

The results of this study provide new information on the production *in vitro* of antifungal metabolites by *Trichoderma viride* commonly employed as a biological control agent of plant pathogens. Further studies with viridepyronone are needed to clarify its role in the biocontrol process. In addition, studies on the antifungal activity of viridepyronone in comparison to that of 6-*n*-pentyl-2*H*-pyran-2-one and related metabolites are in progress.

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