Antifungal Metabolites from Trichoderma harzianum

Emilio L. Ghisalberti, and Catherine Y. Rowland

J. Nat. Prod., **1993**, 56 (10), 1799-1804• DOI:
10.1021/np50100a020 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 56, No. 10, pp. 1799-1804, October 1993

ANTIFUNGAL METABOLITES FROM *TRICHODERMA HARZIANUM*

EMILIO L. GHISALBERTI* and CATHERINE Y. ROWLAND

Department of Chemistry, The University of Western Australia, Nedlands 6009, Australia

ABSTRACT.—A detailed examination of the metabolites produced in liquid cultures by a strain of *Trichoderma harzianum*, isolated from wheat roots, has resulted in the identification of a further five metabolites. Two of these, cyclonerodiol [**5**] and the octaketide keto diol **6**, have previously been isolated from a strain of *Trichoderma koningii*. The structures of the three new octaketide-derived compounds **7**, **8**, and **10** have been deduced from spectroscopic and chemical studies. All newly isolated compounds show antibiotic activity towards the take-all fungus, *Gaeumannomyces graminis* var. *tritici*.

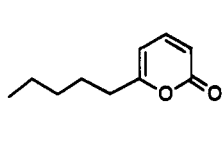
Trichoderma species (Deuteromycotina) have received considerable attention as biocontrol agents of soil-borne plant pathogens involved in the "damping-off" disease of young seedlings. *Trichoderma harzianum* Rifai is perhaps the most studied of the *Trichoderma* species for biocontrol and, arguably, the most effective (1). Circumstantial evidence implicating the action of antibiotic metabolites as a contributing mechanism in biocontrol is increasing (1). In previous work, we have examined three isolates of *T. harzianum* and found that they varied in their ability to antagonize the take-all fungus *Gaeumannomyces graminis* (Sacc.) Arx and Oliver var. *tritici* Walker (Ggt) on agar plates and glass house screening tests (2). Isolate 71 (IMI 311090), the most effective in disease suppression, was shown to produce pentyl-α-pyrone [**1**], the octaketide derived acetal diol **2**, the biogenetically related **3** (3,4), and the diterpenoid diketone **4**, the first example of this class of compound isolated from *Trichoderma* species (5). In continuation of this work, we have examined in more detail the other metabolites produced by this strain. This report describes the isolation of five other metabolites and presents evidence for the structures assigned to three new compounds.

RESULTS AND DISCUSSION

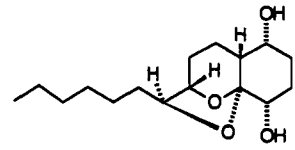
The liquid medium from cultures of *T. harzianum* isolate WU 71 (IMI 311090), grown over a period of 3 months, was extracted with EtOAc as described previously (3,4). Tlc analysis of the crude extract showed that, apart from compounds **1–4**, whose structure and occurrence in this strain of *Trichoderma* has previously been described (3–5), at least five other compounds were present. The components were isolated by radial plate chromatography (rpc) and are described in order of elution.

The first compound obtained after the elution of harziandione [**4**] showed spectral properties which corresponded to those reported (6,7) for cyclonerodiol [**5**]. This sesquiterpene diol, isolated from, inter alia, *Gibberella fujikuroi* and *Trichoderma koningii*, has been shown to have plant-growth-regulating activity (6). It significantly inhibited the growth of etiolated wheat coleoptiles (61% at 10^{-3} M) but did not show significant antibacterial or antifungal activity. It is worth noting that in *T. koningii* cyclonerodiol [**5**] does not appear to accumulate. Analysis of fermenting cultures of *T. koningii* showed the diol to be present after 8 days' incubation, but it could not be detected in the medium after 14 days.

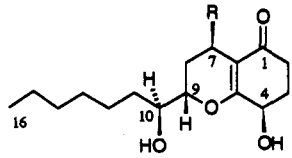
The second and third compounds eluted were identical to the acetal diol **2** and the keto diol **6** previously described (4). This last compound has been found in extracts from cultures of *T. koningii* (8). Since it co-occurs with the acetal diol **2** and generated from it under mild acidic conditions (4), part of it may arise as an artifact.



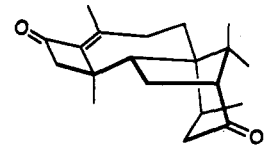
1



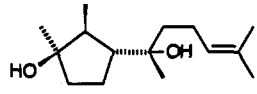
2



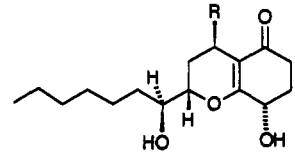
3 R=OH
7 R=H



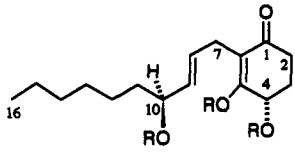
4



5



6 R=H
8 R=OH



9 R=Ac
10 R=H

The next compound isolated was **7**, obtained as a crystalline material which showed $[M]^+$ 282 and appeared to be isomeric with the keto diol **6** ($C_{16}H_{26}O_4$) (4). Comparison of spectral parameters confirmed this. In particular, the ^{13}C-nmr spectra differed significantly only in the chemical shifts of those carbons assignable to the cyclohexenone ring (Table 1). Thus, C-4 in **6** resonated at δ 65.9, whereas the same carbon in **7** resonated at δ 71.0. Furthermore, in the ^{1}H-nmr spectrum, H-4 appeared as a dd at δ 4.4 with J=4 and 6 Hz compared to the same hydrogen in **6** which showed J values of 5.5 and 12.5 Hz. Thus the hydroxyl group is pseudoequatorial in **6** and pseudoaxial in **7**. To eliminate the possibility that **6** and/or **7** might in fact contain the secondary hydroxyl at C-2, a deuterium exchange study was carried out on the two isomers. In each case, up to two hydrogens could be exchanged for deuterium under basic conditions, indicating that the ketone group was flanked by a methylene group. The amount of exchange was monitored by ^{1}H-nmr spectroscopy by comparing the relative integral areas for the oxymethine proton and the signals assigned to the methylene protons α to the carbonyl (at C-2). The C-2 protons of **6** underwent exchange at an appreciably slower rate than the corresponding protons in **7**. This confirms the 1,4 relationship of the ketone and hydroxyl groups in **6** (4) and establishes this arrangement for **7**. Therefore the new compound has the structure shown in **7** and is the 4β-epimer of **6**.

TABLE 1. ^{13}C-nmr Data of Selected Compounds.

Carbon	Compound				
	6	**7**	**3**	**8**	**9**[a]
C-1	198.1	197.7	195.8	198.3	192.7
C-2	27.1	33.3	33.3	27.1	27.3
C-3	29.0	29.0	28.8	28.6	26.1
C-4	71.0	65.9	65.7	71.0	72.7
C-5	171.3	169.6	171.5	173.5	170.4
C-6	109.1	111.2	113.9	112.6	125.7
C-7	17.6	17.5	57.1	57.2	26.5
C-8	22.7	22.8	31.7	31.7	130.0[b]
C-9	80.8	81.3	77.7	76.8	129.0[b]
C-10	73.2	73.4	73.2	72.8	74.4
C-11	32.8	32.5	32.2	33.1	34.3
C-12	25.4	25.0	25.1	25.4	25.1
C-13	29.2	29.2	29.2	29.2	29.0
C-14	31.7	31.7	32.2	31.1	31.7
C-15	22.6	22.6	22.6	22.6	22.6
C-16	14.1	14.0	14.0	14.1	14.1

[a]Other signals: acetate carbons δ 170.1, 167.2, 164.6, 21.3, 20.9, 20.8.
[b]Assignments are interchangeable.

The next fraction eluted was a mixture of two compounds which were resolved by repeated vlc. The major component of this mixture was identified as the keto triol **3** (3) by comparison with an authentic sample. The minor component **8** was shown to be the corresponding C-4 epimer and showed the same relationship to **3** as **6** did to **7**. The ^{13}C-nmr spectrum of **8** included the signal for C-4 (δ 71.0, d) at a chemical shift identical to that of the corresponding carbon in the spectrum of **6** (Table 1).

The most polar fraction eluted was a mixture of several compounds. Acetylation and further fractionation resulted in the isolation of a homogeneous compound whose structure **9** was deduced as follows. The ^{13}C-nmr spectrum exhibited resonances for twenty-two carbon atoms, six of which were associated with acetyl groups (δ 170.1, 167.2, 164.6, each s; 21.3, 20.9, 20.8, each q) which appeared as sharp signals in the ^{1}H-nmr spectrum at δ 2.22, 2.18, and 2.03. That two were associated with a secondary oxygen was evidenced by the occurrence of doublets at δ 74.4 and 72.7. The presence of a β-acetoxy-α,β-unsaturated ketone was inferred from the chemical shifts of sp^2-carbons, which appeared as singlets at δ 192.7 (carbonyl), 125.7 (α carbon), and 170.4 (β carbon). Taking into account the multiplicity of the carbon signals in the ^{13}C-nmr spectrum (4d, 8t, 4q), the proton count totalled 32, thus yielding a molecular formula of $C_{22}H_{32}O_7$ (mol wt 408). The highest mass peak in the ms of **9** was observed at *m/z* 323, a difference of 85 amu (C_6H_{13}) from the projected mol wt. This loss can be accommodated by considering that the ^{13}C-nmr spectrum shows signals consistent with the presence of an *n*-hexyl moiety. Extensive ^{1}H-^{1}H decoupling revealed that an acetoxymethine proton (H-10, δ 5.15, ddd) was coupled (J=6.8 Hz) to methylene protons (H_2-11, δ 1.5 and 1.6) of an alkyl group, on one side, and a vinylic proton (H-9, δ 5.38, dd, J=7, 15 Hz) of a trans-disubstituted double bond on the other. In addition, the second vinylic proton (H-8, δ 5.55, ddt) showed vicinal coupling (J=6 Hz) to a doubly allylic methylene group (H_2-7, δ 2.9) and long range coupling (J=1 Hz) to the acetoxymethine proton. This establishes the connectivity between C-7–C-16 in structure **9**. The C-4–C-2 connectivity was shown from that fact that H-4 (δ 5.39) appeared as a dd (J=5.5, 12.5 Hz) with coupling to methylene protons (δ 2.1–2.3) which, in turn, were coupled to two

geminal protons at δ 2.85 (ddd, J=18, 12, 6 Hz) and 2.60 (J=18, 5.5, 3 Hz). Furthermore, irradiation of the signals for the doubly allylic methylene protons led to a sharpening of the multiplet of the C-4 acetoxymethine proton, establishing the allylic nature of C-4 and C-7.

The relative stereochemistry at C-4 was assigned as shown, since the chemical shifts for C-1–C-4 (δ 192.7, 27.3, 26.1, 72.7) in **9** corresponded more closely to those observed for the same carbons (δ 192.0, 27.1, 26.3, 72.3) in the acetate derivative of **6** (α-epimer) than to those (δ 194.8, 31.6, 26.4, 66.5) of the acetate derivative of **3** (β-epimer) (4). The relative stereochemistry at C-10 was taken to be that observed for the other members of this series.

The remainder of the acetylated fraction, although homogeneous by tlc, contained several compounds, since the ^{1}H-nmr spectrum exhibited up to 10 signals in the acetyl group region. Presumably, this mixture reflects the presence of species epimeric at C-4 as well as acetylated tautomers of the original β-hydroxy-α,β-unsaturated ketone functionality, and the isolation of one of them (**10**) as the triacetate **9** was fortuitous.

It should be pointed out that attempts to secure the absolute configuration for this group of octaketide metabolites have been unsuccessful so far. Therefore, for structures **2**, **3**, **6–9** only the relative stereochemistry is implied.

The biosynthesis of the octaketide metabolites of *T. harzianum* poses some interesting questions. For example, oxygenation at C-7, the site carrying a carbonyl group in a presumed polyketide precursor, is not always retained. On the other hand, oxygenation at C-4 is presumably introduced with lack of stereoselectivity. This suggests the operation of parallel but different biosynthetic pathways. The origin of the array of monocyclic triols (*e.g.*, **10**) is not clear, although it is tempting to consider them as possible precursors of **2** and **6**. Whether the tautomer trapped as the triacetate **9** is an anabolic or catabolic metabolite, its presence can be taken to suggest that counterparts to **3**, **7**, and **8** are also produced.

The isolation of the pyrone **1**, the octaketide metabolites **2**, **3**, **6**, and cyclonerodiol [**5**] from *T. harzianum*, isolate 71, and of **1** (9,10), **2** (11), **3** (3), **5** (6), and **6** (8) from isolates of *T. koningii* points to a chemotaxonomic relationship between these two species. On the other hand, chemotaxonomically distinct strains of *T. harzianum* are also known (2). Thus isolate 70 produces two anthraquinones only and is not effective in suppressing the take-all fungus (2), whereas isolate 73 produces two butenolides which appear to have antibiotic activity (4).

All the newly isolated compounds, **5–8** and the triol (**10** and/or its tautomer) from deacetylation of **9**, showed antifungal activity against Ggt comparable to that exhibited by the congeners **2** and **3** (3,4). None, however, significantly inhibited the growth of *Rhizoctonia solani*. Details of this selective antagonism will be published in a separate paper. Cutler *et al.* (11) have shown that the growth of etiolated wheat coleoptiles was inhibited by **2**, **5**, and **6** (57, 61, and 100%, respectively at 10^{-3} M). Interesting also is the observation that strains of *T. harzianum* that are effective as biocontrol agents also retard the germination of lettuce seedlings. However, following germination the seedlings are free from pathogens and there appears to be growth-enhancing effect associated with the presence of *T. harzianum* (12). What role the *Trichoderma* metabolites play in one or more of these activities remains to be established.

EXPERIMENTAL

GENERAL EXPERIMENTAL METHODS.—^{1}H-nmr and ^{13}C-nmr spectra were recorded at 300 MHz and 75 MHz, respectively, on a Brucker AM-300 Spectrometer. Mass spectra were measured with a Hewlett-Packard 5986 GC/MS System (35 eV). [α]D was measured in $CHCl_3$ using a Perkin-Elmer 141 Polarimeter with a 1 dm cell. For tlc, Kieselgel $60F_{254}$ aluminum sheets (Merck) were used. Rpc was carried out using

a Chromatotron Model 7924T (Harrison Research, Palo Alto, California) with 1-mm layers of Kieselgel 60 PF_{254} gipshaltig (Merck Art. 7749) spread on glass plates under a N_2 atmosphere. For vlc, silicic acid 100 mesh (Mallinckrodt) packed in a sintered glass funnel was used. Preparation of liquid cultures and bioassay methods have been described before (3).

ISOLATION OF METABOLITES FROM THE LIQUID CULTURE OF *T. HARZIANUM*.—The liquid medium (10 liters) of a 3-month-old culture of *T. harzianum* WU 71 (culture deposited with the Commonwealth Mycological Institute IMI 311090) was extracted repeatedly with EtOAc, and the combined organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The oily extract (1.26 g) by tlc analysis (Si gel; 20% hexane: 80% EtOAc) appeared to contain at least nine compounds. Rpc using gradient elution (petroleum ether to EtOAc) gave nine fractions. Fraction 2 (84 mg) yielded harziandione (**4**], R_f 0.84 [EtOAc-hexane (4:1)], which has been previously reported (5). Fraction 3 (20 mg) was purified by cc on Si gel to give an oil, R_f 0.74 [EtOAc-hexane (4:1)], which was identical to cyclonerodiol [**5**] (6,7): $[\alpha]^{25}D$ −20° (c=0.1; $CHCl_3$) [lit. (7) $[\alpha]D$ −21° (c=1.04, $CHCl_3$)]. Fraction 4 (30 mg) yielded a solid, R_f 0.6 [EtOAc-hexane (4:1)], identical to the acetal diol **2** (4). Fraction 5 (40 mg) yielded a crystalline material, R_f 0.5 [EtOAc-hexane (4:1)], which was shown to be the keto diol **6** (4). Fraction 6 (74 mg) was further purified by rpc, gradient elution from CH_2Cl_2 to EtOAc, to give a crystalline sample of the keto diol **7**, R_f 0.4 [EtOAc-hexane (4:1)]. Fraction 7 (230 mg) was semicrystalline and contained two compounds, R_f 0.35 and 0.30 [EtOAc-hexane (4:1)], which were separated by vlc [elution with EtOAc-CH_2Cl_2 (1:1)] to give the new keto triol **8** (7 mg) and the 4-epimer **3**, identical with an authentic sample (3). Fractions 8 and 9 appeared to contain the same compounds, R_f 0.2 and 0.1 [EtOAc-hexane (4:1)]. The two fractions were combined and treated with Ac_2O/pyridine overnight. The product (350 mg) recovered by the usual workup was subjected to vlc using Si gel. Gradient elution from hexane to CH_2Cl_2 afforded two fractions. The more polar component (80 mg), R_f 0.41 [EtOAc-CH_2Cl_2 (4:1)], appeared from the ^{1}H-nmr spectrum to be a mixture, whereas the minor component, R_f 0.87 [EtOAc-CH_2Cl_2 (4:1)] (20 mg) was a homogeneous sample of the triacetate **9**. A small sample of **9** in MeOH was deacetylated by treatment with aqueous $NaHCO_3$ over two days. The triol **10** recovered with EtOAc was used for testing in the antibiotic assay.

Keto diol **7**.—Recrystallized from Et_2O/diisopropyl ether as needles: mp 94–96°; $[\alpha]^{25}D$ +107° (c=1.2); ^{1}H nmr δ 4.40 (1H, dd, J=6, 4 Hz, H-4), 3.77 (1H, ddd, J=11, 7.5, 2 Hz, H-9), 3.63 (1H, td, J=7.5, 2 Hz, H-10), 2.60 (1H, ddd, J=17, 7, 5 Hz, H_A-2), 2.45 (1H, ddd, J=16.5, 5.5, 2 Hz, H-3), 2.35 (1H, ddd, J=17, 9, 5 Hz, H_B-2), 0.87 (3H, t, J=6.5 Hz, H_3-16); ^{13}C nmr see Table 1; eims *m/z* (rel. int.) $[M]^+$ 282 (9), 264 (4), 179 (5), 168 (13), 155 (52), 150 (47), 142 (37), 141 (16), 140 (15), 139 (14), 137 (22), 124 (34), 109 (24), 43 (100).

Keto triol **8**.—Needles: mp 98–100°, $[\alpha]^{25}D$ +89° (c=0.9); ^{1}H nmr δ 4.80 (1H, br s, $W_{h/2}$ 8 Hz, H-4), 4.07 (1H, m, H-9), 3.79 (1H, br s, $W_{h/2}$ 7 Hz, H-7), 3.68 (1H, apparent q, J=6.5 Hz, H-10), 2.7–2.5 (3H, H_2-2 and H_A-3), 0.88 (3H, t, J=6.4 Hz, H_3-16); ^{13}C nmr see Table 1; eims *m/z* (rel. int.) $[M-18]^+$ 280 (22), 262 (6), 209 (6), 195 (5), 181 (10), 170 (11), 166 (14), 165 (18), 155 (49), 141 (23), 124 (39), 113 (59), 111 (33), 85 (36), 43 (100).

Triacetate **9**.—Oil: $[\alpha]^{25}D$ +30.6° (c=1.5); ^{1}H nmr δ 5.55 (1H, ddt, J=15, 6, 1 Hz, H-8), 5.39 (1H, dd, J=12.5, 5.5 Hz, H-4), 5.38 (1H, dd, J=15, 7 Hz, H-9), 5.15 (1H, ddt, J=7.1, 6.8 Hz, H-10), 2.90 (2H, m, H_2-7), 2.85 (1H, ddd, J=18, 12, 6 Hz, H_A-2), 2.60 (1H, ddd, J=18, 5.5, 6 Hz, H_B-2), 2.3–2.1 (2H, m, H_2-3), 2.22, 2.18, and 2.03 (each 3H, s, acetoxymethyl protons), 1.6–1.5 (2H, m, H_2-11), 0.87 (3H, t, J=6.5 Hz, H_3-16); ^{13}C nmr see Table 1; eims *m/z* (rel. int.) $[M-85]^+$ 323 (2), 306 (6), 288 (3), 264 (10), 246 (20), 175 (11), 135 (18), 85 (14), 43 (100).

DEUTERIUM EXCHANGE EXPERIMENTS ON THE EPIMERIC KETO DIOLS **6** AND **7**.—Solutions of the keto diols (5 mg) in MeOH-d_4–D_2O (8:2) (0.5 ml) were individually treated with a solution of Na_2CO_3 in D_2O (1 mg in 10 μl) and left for 3 weeks at 22°. The products recovered in the normal way were analyzed by ^{1}H-nmr spectroscopy and ms. The sample from the keto diol **7** was shown to have incorporated two deuterium atoms; the ms included a molecular ion peak at *m/z* 284 and the following ions: *m/z* 266, 170, 157, 152, 144, which had counterparts 2 amu lower in the ms of **7**. The ^{1}H-nmr spectrum lacked signals for the C-2 protons at δ 2.60 and 2.35. The sample from the keto diol **6** showed peaks at *m/z* 283 and 284 with an isotope enhancement of 37%-$^2H_1$ and 20%-$^2H_2$. Paired fragment ions were observed at *m/z* 266/264, 211/209, 155/153. The ^{1}H-nmr spectrum of the sample showed no reduction in the integral area of the signals for the hydroxymethine proton when compared to the area of the other hydroxymethine protons, but rather a reduction (ca. 50%) and change in multiplicity for the signals at δ 2.6 and 2.5 due to the C-2 protons.

ACKNOWLEDGMENTS

We gratefully acknowledge support of this work by a grant from the Australian Research Council.

LITERATURE CITED

1. E.L. Ghisalberti and K. Sivasithamparam, *Soil Biol. Biochem.*, **23**, 1011 (1991).
2. E.L. Ghisalberti, M.J. Narbey, M.M. Dewan, and K. Sivasithamparam, *Plant Soil*, **121**, 287 (1990).
3. R.W. Dunlop, A. Simon, K. Sivasithamparam, and E.L. Ghisalberti, *J. Nat. Prod.*, **52**, 67 (1989).
4. F. Almassi, E.L. Ghisalberti, M.J. Narbey, and K. Sivasithamparam, *J. Nat. Prod.*, **54**, 396 (1991).
5. E.L. Ghisalberti, D.C.R. Hockless, C. Rowland, and A.H. White, *J. Nat. Prod.*, **55**, 1690 (1992).
6. H.G. Cutler, J.M. Jacyno, R.S. Phillips, R.L. Von Tersch, P.D. Cole, and N. Montmemurro, *Agric. Biol. Chem.*, **55**, 243 (1991).
7. D. Laurent, N. Goasdone, F. Kohler, F. Pellegrin, and N. Pletzer, *Magn. Reson. Chem.*, **28**, 662 (1990).
8. H.G. Cutler, D.S. Himmelsbach, B. Yagen, R.F. Arrendale, J.M. Jacyno, P.D. Cole, and R.H. Cox, *J. Agric. Food Chem.*, **39**, 977 (1991).
9. H. Benoni, K. Taraz, H. Korth, and G. Pulverer, *Naturwissenschaften*, **77**, 539 (1990).
10. A. Simon, R.W. Dunlop, E.L. Ghisalberti, and K. Sivasithamparam, *Soil Biol. Biochem.*, **20**, 263 (1987).
11. H.G. Cutler, D.S. Himmelsbach, R.F. Arrendale, P.D. Cole, and R.H. Cox, *Agric. Biol. Chem.*, **53**, 2605 (1989).
12. J.M. Lynch, K.L. Wilson, M.A. Ousley, and J.M. Whipps, *Lett. Appl. Microb.*, 59 (1991).

Received 8 April 1993