Structure and Biosynthesis of Harzianopyridone, an Antifungal Metabolite of *Trichoderma harzianum*

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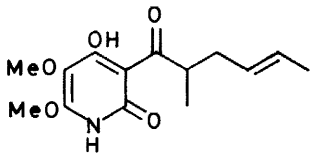
An antifungal metabolite of *Trichoderma harzianum* has been shown to be (*E*)-4-hydroxy-5,6-dimethoxy-3-(2-methyl-1-oxohex-4-enyl)pyridin-2-one. The incorporation of [1-^{13}C]- and [1,2-$^{13}C_2$]-acetic acid and [Me-^{13}C]methionine into harzianopyridone and the sites of labelling were established by n.m.r. methods. A low incorporation of [U-^{14}C]aspartic acid was also observed.

The fungus, *Trichoderma harzianum*, has been the subject of considerable study for use in the biological control of plant pathogens involved in the 'damping-off' diseases of young seedlings.[1-3] The volatile metabolite, 6-pentyl-α-pyrone is responsible for some of this activity.[4] In this paper we describe the evidence for the structure and biosynthesis of a further antifungal metabolite, harzianopyridone (**1**) which is formed in small amounts (*circa.* 1—5 mg/l culture broth) when the fungus is grown on a 2% malt extract medium in surface culture for 30 days.

Harzianopyridone, $C_{14}H_{19}NO_5$ (M^+, 281.127), possessed broad i.r. absorption in the range 3 100—2 600 cm^{-1} and sharper bands at 1 725, 1 650, 1 600, and 720 cm^{-1}. It had u.v. absorption at 243, 267, and 331 nm. As isolated, harzianopyridone showed no reproducible optical activity. Small scale experiments showed that the compound gave a dihydro derivative, (M^+, 283.143), a diacetate (M^+, 365.150) and on treatment with diazomethane, a dimethyl ether (M^+, 309). It gave a strong wine-red colour with ferric chloride. The mass spectrum of harzianopyridone possessed a base peak at 198.042 a.m.u. corresponding to the loss of a C_6H_{11} fragment. The



(1)

nature of this fragment [C(18)–C(13)] followed from the partial structure derived from the ^{1}H n.m.r. spectrum (see Table 1).

The ^{1}H n.m.r. spectrum revealed the presence of two methyl groups attached to methine carbons, one of which was an alkene. There were also resonances attributable to two methoxy groups, a *trans* disubstituted alkene and further allylic proton resonances. A signal at δ 16.4 was assigned to an acidic hydrogen and a broad signal at δ 12.3 to an amide NH. Spin decoupling studies established the presence of the grouping $CH_3CH{=}CHCH_2CH(CH_3)C{=}O$. Irradiation of the methyl doublet at δ 1.65 modified the alkene AB multiplets at δ 5.42 (removing a 6.5 Hz coupling) and 5.38 (removing a 1.5 Hz coupling). Small couplings were also removed from the allylic proton signals at δ 2.05 and 2.45. Analysis of these allylic signals which were geminally coupled (*J* 14 Hz) showed that they were also coupled (*J* 6.5 Hz) to a single proton sextet at δ 3.95. This signal also showed coupling to the other methyl group (δ 1.30).

Whilst the spectroscopic data were consistent with an alkyl 4-hydroxypyridone formulation, the overall structure and in particular the relative disposition of the functional groups around the pyridone ring, were firmly established by an *X*-ray crystallographic structure determination (see Figure 1).

Table 1. The ^{1}H n.m.r. signals for harzianopyridone determined in $CDCl_3$ at 360 MHz

Signal	Integral	Multiplicity and coupling constants (Hz)	Assignment
1.30	3	d (6.5)	9-H
1.65	3	d (6.5)	13-H
2.05	1	d (14) of t (6.5)	10-H
2.45	1	d (14) of br t (6)	10-11
3.79	3	s	15[a]-H
3.95	1	sextet (6.5)	8-H
4.17	3	s	14-H
5.38	1	overlapping, d (14.5), dd (6.5) multiplets	11-H, 12-H
5.42	1		
12.3	1	br	NH
16.4	1	s	OH

[a] Assigned by an n.O.e. effect (2.4%) between this signal and the OH signal.

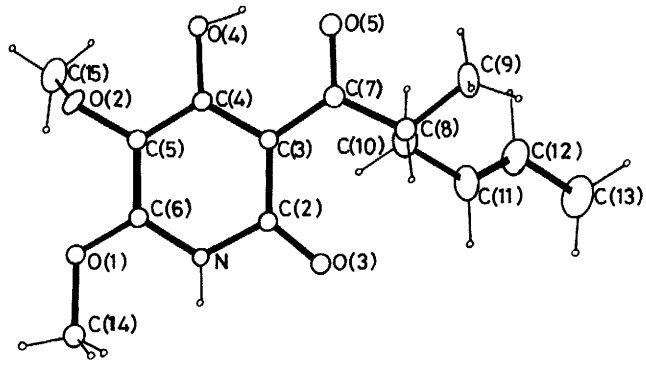


Figure 1. The crystal structure of harzianopyridone

This structure possesses a chiral centre at C-8. However harzianopyridone behaved as a racemate and it is probable that racemization of this enolizable centre occurred during the work-up.

As a prelude to biosynthetic studies, the ^{13}C n.m.r. signals were assigned (see Table 2). Only 13 of the 14 ^{13}C n.m.r. signals were observed at room temperature. The remaining signal (δ 173) was detected at −25 °C. Whilst the majority of the ^{13}C n.m.r. signals could be assigned on the basis of their chemical

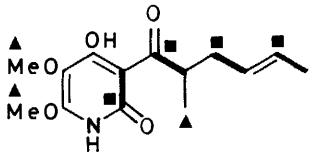
Table 2. The ^{13}C n.m.r. signals and biosynthetic results for harzianopyridone (determined in $CDCl_3$ at 90.55 MHz)

			Biosynthetic results		
				Enrichments[a]	
Signal	hydrogens	Attached Assignment	J (from [1,2-$^{13}C_2$]acetate)/Hz	[1-^{13}C]Ac	Me-met.
16.34	CH_3	C-9			5.6
17.94	CH_3	C-13	41.6		
36.14	CH_2	C-10	42.0	4.2	
43.17	CH	C-8	39.8		
57.55	CH_3	C-15[b]			8.9
61.53	CH_3	C-14[b]			8.4
100.59	C	C-3	75.2		
121.58	C	C-5			
127.07	CH	C-12	43.0	4.9	
128.68	CH	C-11	43.8		
155.71	C	C-6			
161.77	CO	C-2	76.13	4.7	
172.92	COH	C-4			
209.77	CO	C-7	40.8	4.6	

[a] $\text{Enrichment} = \frac{\text{enriched sample peak height}}{\text{nat. abund. sample peak height}} \times \text{normalization factor.}$

$\text{Normalization factor} = \frac{\text{total peak height unlabl. signals. nat. abund. spec.}}{\text{total peak height unlabl. signals enriched spec.}}$

[b] Assignment may be interchanged.



[1,2-$^{13}C_2$]Acetate units
Enriched by [1-^{13}C]acetate
Enriched by [Me-^{13}C]methionine

Figure 2. The biosynthesis of harzianopyridone

shift and the number of attached hydrogen atoms, the signals for C-2 and C-4 of the pyridone were distinguished by the heteronuclear long-range coupling which was observed between the OH signal at δ 16.4 and the ^{13}C signals at δ 100.59 (C-3) and δ 173.00 (C-4).

The polyketide origin of the side chain was established by [1-^{13}C]- and [1,2-$^{13}C_2$]-acetate feeding experiments. These afforded harzianopyridone (**1**) with the enrichment and coupling pattern shown in Figure 2. Hence the molecule contains a tetraketide unit. The origin of the extra carbon atom in the side chain and of the methoxy groups was established by [Me-^{13}C]methionine feeding experiments when the signals at δ 16.34, 57.55, and 61.53 showed a significant enrichment.

These biosynthetic results are similar to those of tenellin,[5,6] and ilicicolin H[7] in which the side chains are also derived from acetate units. In these cases the remainder of the pyridone ring is derived from phenylalanine. In nicotinic acid biosynthesis[8] aspartic acid can fulfil this role. [U-^{14}C]Aspartic acid was incorporated into harzianopyridone by *Trichoderma harzianum* but only to the extent of 0.03%. Insufficient material was available to devise a degradation to demonstrate that this incorporation was restricted to the pyridone ring. Its level is below that at which a carbon-13 n.m.r. experiment would be meaningful and hence this result is only indicative of a possible precursor.

When bioassayed against a range of phytopathogenic fungi, harzianopyridone showed significant activity against *Botrytis cinerea* and *Rhizoctonia solani*. Fungal pyridones are relatively rare and this metabolite is the first example of this type to have been obtained from a *Trichoderma* species.

Experimental

General.—^1H and ^{13}C n.m.r. spectra were determined in deuteriochloroform solution at 360 and 90.55 MHz respectively on a Bruker WH 360 spectrometer. I.r. spectra were for Nujol mulls and were determined on a Perkin-Elmer 597 spectrometer. U.v. spectra were determined in ethanol on a Pye Unicam SP 800 spectrometer. Light petroleum refers to the fraction, b.p. 60—80 °C. Solutions were dried over sodium sulphate. Silica gel (Merck 9385) was used for chromatography. *Trichoderma harzianum* was maintained on malt extract agar slopes. This strain has been deposited in the culture collection of the Commonwealth Mycological Institute, Kew, as IMI 298371.

Isolation of Harzianopyridone.—*Trichoderma harzianum* was grown in surface culture on a 2% malt medium in Roux bottles (200 ml medium per bottle) for 30 days. The mycelium was filtered off and the broth was extracted with ethyl acetate. The extract was dried and the solvent was evaporated to give a gum. This was chromatographed first on silica gel 60 using 3% methanol–dichloromethane as eluant. The fractions which contained harzianopyridone (t.l.c.) were rechromatographed on silica using 20% ethyl acetate–light petroleum as eluant. On occasions further purification by preparative layer chromatography (p.l.c.) on silica (eluant, 50% ethyl acetate–light petroleum) was also necessary. *Harzianopyridone* (**1**) (*circa.* 1—5 mg. litre^{-1} culture broth) crystallized from ethyl acetate–light petroleum as needles, m.p. 125 °C (Found: C, 59.3, 59.6; H, 7.00, 7.11; N, 4.92, 4.95; $C_{14}H_{19}NO_5$ requires C, 59.8; H, 6.81; N, 4.97%); $\nu_{max.}$(Nujol) 3 100—2 600br, 1 725, 1 650, 1 600 and 720 cm^{-1}; *m/z* 281 (M^+, 20%) 263 (8), 248 (8), 198 (100), and 171 (8); (Found: 281.127; $C_{14}H_{19}NO_5$ requires 281.126; Found: 198.042; $C_{14}H_{19}NO_5 - C_6H_{11}$ requires 198.040). The ^1H and ^{13}C n.m.r. data are tabulated.

Derivatives of Harzianopyridone.—(*a*) Harzianopyridone (50 mg) in ethyl acetate (20 ml) containing 10% palladium on

Table 3. Fractional atomic co-ordinates ($\times 10^4$) with estimated standard deviations in parentheses

Atom	*x*	*y*	*z*
	2 425(3)	−1 325(2)	666(2)
O(1)	3 816(2)	−3 483(1)	365(2)
O(2)	7 365(2)	−3 481(2)	1 309(2)
O(3)	910(2)	715(1)	975(2)
O(4)	7 757(2)	−1 292(2)	2 107(2)
O(5)	6 302(2)	951(2)	2 528(2)
C(2)	2 450(3)	−186(2)	1 120(2)
C(3)	4 307(3)	−176(2)	1 677(2)
C(4)	5 963(3)	−1 270(2)	1 681(2)
C(5)	5 770(3)	−2 397(2)	1 263(2)
C(6)	3 978(3)	−2 395(2)	755(2)
C(7)	4 565(3)	909(2)	2 239(2)
C(8)	2 795(4)	1 934(2)	2 577(2)
C(9)	3 471(5)	3 191(3)	2 716(3)
C(10)	1 780(5)	1 208(3)	3 775(3)
C(11)	−166(5)	2 015(3)	4 189(3)
C(12)	−434(6)	2 514(3)	5 168(3)
C(13)	−2 376(6)	3 283(4)	5 614(3)
C(14)	1 951(4)	−3 539(2)	−159(3)
C(15)	7 552(5)	−4 386(3)	2 534(3)

charcoal (500 mg) was stirred under hydrogen for 3 h. The mixture was filtered through Celite and the solvent was evaporated to afford the *dihydro* derivative as a gum (36 mg) (Found: M^+, 283.143. $C_{14}H_{21}NO_5$ requires M, 283.142); $\nu_{max.}$ 3 000br, 1 725, 1 605 cm^{-1}; δ_H($CDCl_3$; 90 MHz) 1.01 and 1.19 (each 3 H, br), 3.67 (3 H, s, OMe), 3.91 (1 H, m), and 4.05 (3 H, s, OMe); *m/z* 283.241, 198, and 168.

(*b*) Harzianopyridone (50 mg) in methanol (10 ml) was treated with an excess of ethereal diazomethane at 0 °C overnight. The solvent was evaporated and the residue was purified by p.l.c. on silica to give the dimethyl derivative as a gum (M^+, 309. $C_{16}H_{23}NO_5$ requires M^+, 309); $\nu_{max.}$ 1 725 and 1 645 cm^{-1}; δ_H($CDCl_3$; 90 MHz) 1.10 and 1.56 (each 3 H, br s), 3.69, 3.92, 3.97, and 4.12 (each 3 H, s), and 5.41 (2 H, m).

(*c*) Harzianopyridone (50 mg) in pyridine (2 ml) was treated with acetic anhydride (2 ml) overnight. The mixture was diluted with ethyl acetate, washed with saturated aeueous copper sulphate, aqueous sodium hydrogen carbonate, and dried. The solvent was evaporated and the residue was purified by preparative layer chromatography on silica to afford the *diacetate* (42 mg) as a gum (Found: M^+, 365.150. $C_{18}H_{23}NO_7$ requires M, 365.147); $\nu_{max.}$ 1 725, 1 720, and 1 650 cm^{-1}; δ_H 1.05 and 1.53 (each 3 H, br s), 2.14 and 2.17 (each 3 H, s, OAc), 3.71 and 3.89 (each 3 H, s, OMe), and 5.30 (2 H, m).

Biosynthetic Experiments.—(*a*) 2% Malt extract medium (15 l) was distributed between 20 2 l conical flasks, sterilized and inoculated with *T. harzianum*. After 14 days growth, sodium [1-^{13}C]acetate (1 g) in distilled water (20 ml) was evenly distributed between the flasks. After a further 14 days growth the fermentation was harvested and the harzianopyridone (20 mg) was isolated and purified as above and examined by ^{13}C n.m.r. (see Table 2).

(*b*) The experiment was repeated with sodium [1,2-$^{13}C_2$]acetate (1 g) and with [methyl-^{13}C]methionine (1 g) in saturated aqueous sodium hydrogen carbonate (20 ml).

(*c*) The above experiment was repeated with [L-U-^{14}C]-aspartic acid (50 μC) in distilled water (20 ml). The resulting harzianopyridone had 34 779 d.p.m. (0.03% incorporation).

Crystallographic Data.—$C_{14}H_{19}NO_5$, M = 281.3, triclinic, space group *PT*, a = 6.722(1), b = 10.386(2), c = 10.882(2) Å, α = 75.55(2), β = 85.31(2), γ = 77.92(2)°, U = 718.9 Å^3, Z = 2, D_c = 1.30 g cm^{-3}. Monochromated Mo-Kα radiation, λ = 0.71 069 Å, μ = 1.0 cm^{-1}.

A crystal of size *circa.* 0.20 × 0.15 × 0.15 mm was mounted on an Enraf-Nonius CAD 4 diffractometer operating in the θ—2θ mode, $\Delta\theta = (0.8 + 0.35 \tan\theta)°$, with a maximum scan time of 1 min. 2 640 Reflections were measured for $2 < \theta < 25°$, $+h$, $\pm k$, $\pm l$. 1 660 Reflections with $|F^2| > \sigma(F^2)$ were used in the refinement where $\sigma(F^2) = [\sigma^2(I) + 0.04I)^2]^{\frac{1}{2}}/Lp$. There was no crystal decay. No absorption corrections were made. The C, N, and O atoms were located by routine direct methods (MULTAN) and refined anisotropically by full matrix least squares. The H atoms were located on a difference map and refined isotropically. The weighting scheme was $w = 1/\sigma^2(F)$. The final R factors were R = 0.047, R' = 0.056. A final difference map was featureless. The programs were from the Enraf-Nonius SDP-Plus package and were run on a PDP 11/34 computer. The final atomic co-ordinates are given in Table 3.The intramolecular distances, bond angles, torsional angles hydrogen atom co-ordinates, and temperature factors have been deposited with the Cambridge Crystallographic Data Centre.*

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* For details see 'Introduction for Authors (1989),' *J. Chem. Soc., Perkin Trans. 1*, 1989, Issue 1.

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