Studies in Terpenoid Biosynthesis. Part XVI.[1] Formation of the Sesquiterpenoid Trichothecin

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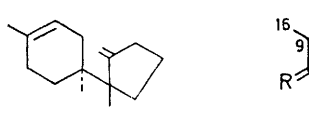
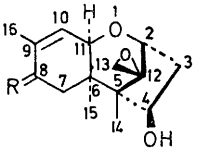
The biosynthetic sequence trichodermol (2) ⟶ 12,13-epoxy-4β,8-dihydroxytrichothec-9-ene (3) ⟶ trichothecolone (4) ⟶ trichothecin (5) in *Trichothecium roseum* has been established. Earlier evidence suggesting the intervention of crotocin (6) has not been substantiated.

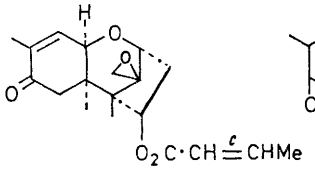
TRICHODIENE (1) has been shown to be a precursor of trichothecolone (4) in *Trichothecium roseum*.[2,3] Although a number of plausible intermediates have been isolated, there is no biosynthetic information on the intervening stages. The introduction of the C-8 carbonyl group of trichothecolone and the order of the late stages in its biosynthesis are the subjects of this paper. We have considered a number of schemes. One attractive hypothesis was that the C-8 and C-11 oxygen functions were related and that they arose by rearrangement of a Δ^9-8,11-epidioxide, itself formed from a ring A diene.[4] There are precedents for the formation of dienes in the related cuprenene series, and this biogenetic scheme might also be reflected in the structure of helicobasidin. A second hypothesis involved the rearrangement of a 7,8-epoxide. A 7,8-epoxide, crotocin (6),[5] co-occurs with trichothecin in *T. roseum*.[6] A third proposal involved simple hydroxylation at C-8 followed by oxidation.[7]



(1)



(2) R = H_2
(3) a; R = α-OH,H
b; R = β-OH,H
(4) R = O



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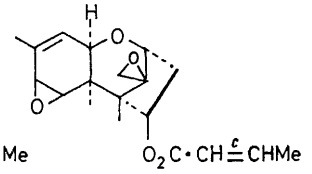

(6)

The introduction of the C-8 carbonyl group prior to the formation of the 2,11-ether linkage of the 12,13-epoxytrichothecene skeleton is implicit in a mechanism involving an 8,11-epidioxide. Trichodermol (2), a metabolite of *Trichoderma* species,[8] lacks a C-8 carbonyl group. It was shown to be present in *Trichothecium roseum* by dilution analysis of a fermentation to which [2-^{14}C]mevalonic acid had been added (0.07% incorporation). [2,10-^{3}H;4,8,14-^{14}C]Trichodermol (2), prepared biosynthetically[9] from [(4*R*)-4-^{3}H;2-^{14}C]mevalonic acid by using *Trichoderma sporulosum*, was incorporated into trichothecin in 3.4% yield by *Trichothecium roseum*. This excluded oxygenation at C-8 prior to formation of the trichothecene skeleton, and showed that, once the skeleton is formed, oxidation at C-4 precedes that at C-8. The retention of the two [(4*R*)-4-^{3}H]mevalonate labels was in accord with the folding of farnesyl pyrophosphate which has been proposed[10] for these compounds.

In the formation of a 7,8-epoxide, a C-5 and a C-2 mevalonoid hydrogen atom are removed from C-7 and C-8, respectively. During the subsequent isomerization of the epoxide to form the carbonyl group, a C-8 hydrogen atom, originating from C-2 of mevalonate, would migrate to C-7. On this basis trichothecin might incorporate four labels from each of the [2-$^3H_2$]- and [5-$^3H_2$]-mevalonates. Our earlier results implied that this was the case.[9] However we have been led to repeat our earlier experiments, with different results (see Table 1). In order to take account of the problems

TABLE 1

Incorporation of [2-$^3H_2$;2-^{14}C]- and [5-$^3H_2$;2-^{14}C]-mevalonates into the metabolites of *T. roseum*

	^{3}H : ^{14}C Ratio	Atom ratio	% Incorporation
[2-$^3H_2$;2-^{14}C]Mevalonic acid	16.56 : 1		
Deoxyrosenonolactone	14.06 : 1	8.00 : 4	3.8
Rosenonolactone	10.41 : 1	5.92 : 4 *	6.1
Trichothecin	7.23 : 1	3.09 : 3 *	1.3
Trichothecolone	7.10 : 1	3.03 : 3 *	
[5-$^3H_2$;2-^{14}C]Mevalonic acid	16.13 : 1		
Deoxyrosenonolactone	16.32 : 1	8.09 : 4	0.81
Rosenonolactone	16.07 : 1	7.97 : 4	1.19
Trichothecin	13.92 : 1	5.17 : 3	0.13
Trichothecolone (i)	10.62 : 1	3.95 : 3	
Trichothecolone (ii)	7.9 : 1	2.89 : 3	

* Calculated on the basis that deoxyrosenonolactone incorporated 8 labels.

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which might arise through the action of prenyl isomerase, the atom ratios of the [2-$^3H_2$]mevalonate experiment are normalized to those of the diterpene, deoxyrosenonolactone. This metabolite was also produced by the fungus and has been shown [11] to incorporate eight [2-$^3H_2$]mevalonate labels. The trichothecin (5) incorporated three [2-$^3H_2$]- and five [5-$^3H_2$]-mevalonoid hydrogen labels. The trichothecin (5) from both feeding experiments was subjected to hydrolysis and exchange in methan[^{2}H]ol containing 2N-sodium deuteroxide. While there was no loss of label by exchange from the [2-$^3H_2$]mevalonate-labelled trichothecolone (4), there was a loss of two [5-$^3H_2$]-mevalonoid labels. The mass spectrum of the deuteriated products showed that two deuterium atoms had been introduced, and the n.m.r. spectrum lacked the resonances at δ 2.31 and 2.89 (*J* 16 Hz) which have been assigned [12] to the 7α- and 7β-protons. The signal due to the other potentially enolisable proton (C-11) at δ 3.85 was unchanged. Thus there are two [5-$^3H_2$]-mevalonoid labels at C-7, excluding a Δ^7-olefin and epoxide from the biosynthesis. The earlier result may have been due to a combination of easy exchange at C-7 and contamination of the trichothecin with rosenonolactone. Rosenonolactone not only incorporates mevalonate more efficiently than trichothecin but also co-crystallizes with it.

The third route to trichothecolone involves hydroxylation of trichodermol at C-8 followed by oxidation. Reduction of trichothecolone with sodium borohydride afforded a separable mixture of the 8α- and 8β-alcohols (3a and b). Previous workers [7] had only obtained one isomer. In view of the similarity of the 7,8-coupling constants, the stereochemistry of these alcohols was assigned on the basis of solvent shifts in the ^{1}H n.m.r. spectrum (see Table 2). The methyl group resonances

TABLE 2

^{1}H N.m.r. solvent shifts for the epimeric 12,13-epoxy-4β,8-dihydroxytrichothec-9-enes

	8α-Alcohol		8β-Alcohol	
Proton	$CDCl_3$	C_5D_5N	$CDCl_3$	C_5D_5N
13-H	2.84, 3.11	2.90, 3.12	2.83, 3.11	2.74, 2.81
14-H	0.85	1.15	0.82	0.92
15-H	1.07	1.37	0.91	1.04

showed larger solvent shifts in the 8α-alcohol (3a), and the β-oriented C-13 epoxide protons were shielded to a greater extent in the 8β-alcohol (3b). [4,8,14-^{14}C]Trichothecolone was reduced with sodium borohydride and an inactive sample was also reduced with [^{3}H]sodium borohydride. The epimers were separated in each case. The labelled alcohols were then mixed in a suitable ^{3}H : ^{14}C ratio to afford [8β-^{3}H;4,8,14-^{14}C]12,13-epoxy-4β,8α-dihydroxytrichothec-9-ene (3a) and [8α-^{3}H;4,8,14-^{14}C]12,13-epoxy-4β,8β-dihydroxytrichothec-9-ene (3b). The alcohols were then incubated with *T. roseum*. Trichothecolone was isolated from both incubations. It showed a 6.6 and 5.6% incorporation, respectively but in neither instance was tritium retained. The comparable efficiency of these incorporations was surprising. However only 12,13-epoxy-4β,8α-dihydroxytrichothec-9-ene (3a) has been isolated [7] from the fungus and it is likely that this is the genuine intermediate. The efficiency of incorporation of the other epimer may be because it is a substrate for the 8α-hydroxylase, *i.e.* for the preceding step in the biosynthesis. Trichothecolone, which co-occurs with trichothecin in *T. roseum*, was converted into trichothecin by the fungus in 27% yield. Hence esterification is probably one of the last stages in the biosynthesis.

EXPERIMENTAL

General experimental details have been described previously.[13] The strains of *Trichothecium roseum* used in this work were CMI50660 and IFO 6157 and they were cultured as described previously.[14]

Dilution Analysis of T. roseum *for Trichodermol.*—[2-^{14}C]Mevalonic acid (250 μCi) in ethanol (10 ml) was distributed amongst six 5-day old surface cultures (750 ml) of *Trichothecium roseum*. After a further twelve days growth, the mycelium was filtered and the broth was extracted with chloroform. The extract was dried and evaporated to give a gum (0.89 g) which was diluted with unlabelled trichodermol (25 mg). The gum was chromatographed on silica to afford trichodermol (19 mg), m.p. 117—118°, 22 590 disint. min^{-1} mg^{-1} (0.07% incorporation).

Incorporation of Trichodermol.—[2,10-^{3}H;4,8,14-^{14}C]Trichodermol [9] (100 mg) (^{3}H : ^{14}C 4.8 : 1; 8.10^3 disint. min^{-1} mg^{-1}) in ethanol (5 ml) was distributed amongst five 5-day old surface cultures (750 ml) of *T. roseum*. After a further 4 weeks growth, the mycelium was filtered and the broth was extracted with chloroform. Evaporation of the solvent gave a gum which was chromatographed on silica to afford trichothecin (90 mg), m.p. 117—118° (^{3}H : ^{14}C, 4.7 : 1; 300 disint. min^{-1} mg^{-1}; 3.4% incorporation).

Incorporation of [2-$^3H_2$;2-^{14}C]- *and* [5-$^3H_2$;2-^{14}C]-*Mevalonic Acids.*—The mevalonates (50 μCi ^{14}C) in ethanol (0.5 ml) were added separately to 3-day old shake cultures (100 ml) of *T. roseum*. After a further ten days, the mycelium was filtered and the metabolites were recovered as above and purified by preparative layer chromatography. The trichothecin and deoxyrosenonolactone fractions were diluted with inactive material (20 mg each) and crystallized to constant activity. The results are given in Table 1.

Deuterium Exchange Reactions.—The trichothecin (30 mg) from the [2-$^3H_2$;2-^{14}C]- and [5-$^3H_2$;2-^{14}C]-mevalonate experiments was taken up in methan[^{2}H]ol (2 ml) and 2N-sodium deuteroxide (1 ml) was added. The solutions were left at room temperature for 3 h. The methanol was removed *in vacuo* and the residue was extracted with ethyl acetate. The trichothecolone was purified by preparative layer chromatography on silica in ethyl acetate–light petroleum (1 : 1). The trichothecolone (i) (23 mg) from the [2-$^3H_2$;2-^{14}C]-mevalonate experiment showed no change in ^{3}H : ^{14}C ratio.

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[13] J. R. Hanson, J. Hawker, and A. F. White, *J.C.S. Perkin I*, 1972, 1892.
[14] B. Achilladelis and J. R. Hanson, *Phytochemistry*, 1968, **7**, 589.

The trichothecolone (21 mg) from the [5-$^3H_2$;2-^{14}C]-mevalonate experiment showed a drop in $^3H : ^{14}C$ atom ratio of 1.20. The exchange was repeated on this material for a further 24 h. The trichothecolone (ii) (9 mg) which was recovered had $^3H : ^{14}C$ 7.9 : 1. The mass spectrum showed *m/e* 248 [Calc. for $C_{15}H_{18}{}^2H_2O_4$: ($M^+ - H_2O$), 248].

Reduction of Trichothecolone.—Sodium borohydride (100 mg) was added to a solution of trichothecolone (200 mg) in methanol (3 ml) over 2 h. The solution was acidified with dilute hydrochloric acid. The products were recovered in ethyl acetate and purified by preparative layer chromatography on silica in ethyl acetate. Elution of the band at R_F 0.7 gave 12,13-epoxy-4β,8α-dihydroxytrichothec-9-ene (140 mg), which crystallized from chloroform as needles, m.p. 155°, $[\alpha]_D$ −57° (*c* 0.15) {lit.,[7] m.p. 190—191° (from acetone–hexane), $[\alpha]_D$ −53°} (Found: C, 67.6; H, 8.5. Calc. for $C_{15}H_{22}O_4$: C, 67.6; H, 8.3%), $\nu_{max.}$ 3 500, 3 400, 1 340, and 1 070 cm^{-1}, δ 0.85 (3 H, s), 1.07 (3 H, s), 1.87 (3 H, s), 1.95—2.22 (3 H, m), 2.52 (1 H, d, *J* 7 Hz), 2.84 and 3.11 (2 H, ABq, *J* 4 Hz), 3.59 (1 H, d, *J* 6 Hz), 3.82 (1 H, d, *J* 6 Hz), 4.13 (1 H, d, *J* 6 Hz), 4.35 (1 H, dd, *J* 4 and 7 Hz), and 5.57 (1 H, d, *J* 6 Hz). Elution of the band at R_F 0.6 gave 12,13-*epoxy*-4β,8β-*dihydroxytrichothec*-9-*ene* (20 mg), which crystallized as needles from ethyl acetate–light petroleum; m.p. 157—158°, $[\alpha]_D$ +35.4° (*c* 0.26) (Found: C, 67.9; H, 8.0%), $\nu_{max.}$ 3 500, 3 400, 1 340, 1 070, and 960 cm^{-1}, δ 0.82 (3 H, s), 0.91 (3 H, s), 1.83 (3 H, s), 1.9—2.2 (3 H, m), 2.58 (1 H, dd, *J* 7 and 16 Hz), 2.83 and 3.11 (2 H, ABq, *J* 4 Hz), 3.54 (1 H, d, *J* 6 Hz), 3.85 (1 H, d, *J* 6 Hz), 4.10 (1 H, d, *J* 7 Hz), 4.27 (1 H, m), and 5.50 (1 H, d, *J* 6 Hz).

Incubation of [8β-^{3}H;4,8,14-^{14}C]12,13-*Epoxy*-4β,8α-*dihydroxytrichothec*-9-*ene with* T. roseum.—The labelled 4β,8α-diols were mixed and recrystallized to give a sample (15 mg) ($^3H : ^{14}C$ 10.03 : 1; 2 × 10^4 disint. min^{-1} mg^{-1} ^{14}C) which was dissolved in ethanol (0.3 ml) and distributed amongst three 3-day old surface cultures (750 ml) of *T. roseum*. After a further 5 days, the broth was extracted and the metabolites were isolated to afford trichothecin (20 mg), m.p. 117—118° ($^3H : ^{14}C$ 0.07 : 1; 1.0 × 10^3 disint. min^{-1} mg^{-1}; 6.6% incorporation).

Incubation of [8α-^{3}H;4,8,14-^{14}C]12,13-*Epoxy*-4β,8β-*dihydroxytrichothec*-9-*ene with* T. roseum.—The labelled 4β,8β-diols were mixed and recrystallized to give a sample (1 mg) ($^3H : ^{14}C$ 18.6 : 1; 1.8 × 10^4 disint. min^{-1} mg^{-1} ^{14}C) which was dissolved in ethanol (0.1 ml) and added to a 3-day old surface culture of *T. roseum*. After a further 5 days, the broth was extracted and the metabolites were isolated to afford trichothecin (20 mg), m.p. 117—118° ($^3H : ^{14}C$ 0.12 : 1; 51 disint. min^{-1} mg^{-1}; 5.6% incorporation).

In a blank experiment, the mixed C-8 alcohols from a sodium borohydride reduction of trichothecin were dissolved in ethanol and shaken in the *T. roseum* culture medium for 5 days. There was no detectable (g.l.c.) formation of trichothecin. (We thank Mr. B. Dockerill for this experiment.)

Incorporation of Trichothecolone into Trichothecin.—[4,8,14-^{14}C]Trichothecolone (14.5 mg; 7 385 disint. min^{-1} mg^{-1}) (prepared biosynthetically from [2-^{14}C]mevalonic acid) in ethanol (3 ml) was distributed amongst three surface cultures (750 ml) of *T. roseum* 10 days after inoculation. After a further 14 days, the mycelium was filtered and the metabolites were isolated. The trichothecin (91 mg), m.p. 115—116°, showed 779 disint. min^{-1} mg^{-1} (6.7% incorporation).

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