Studies in Terpenoid Biosynthesis. Part VIII.[1] The Formation of the Trichothecane Nucleus

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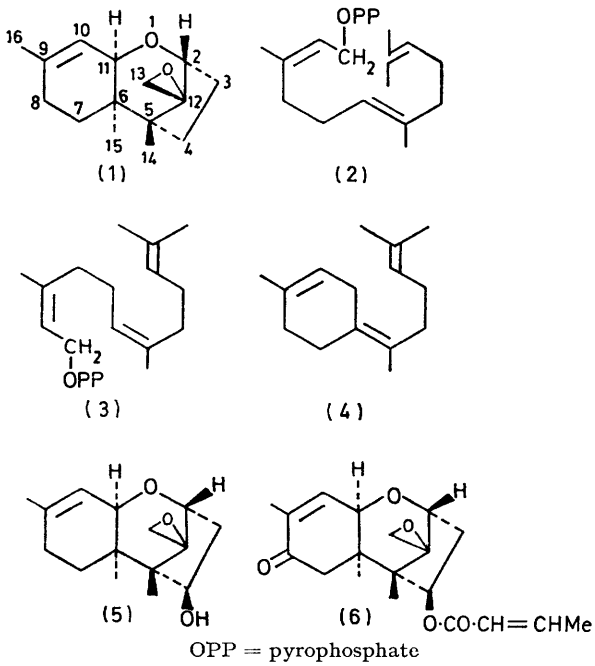
The incorporation of [2-$^3H_2$]-, (4*R*)-[4-^{3}H]-, and [5-$^3H_2$]-mevalonate, [2-^{3}H]geranyl pyrophosphate, and [1-$^3H_2$]- and [2-^{3}H]-farnesyl pyrophosphate into the trichothecane nucleus is described. The results define the manner of folding of the farnesyl pyrophosphate and exclude a γ-bisabolene intermediate in the biosynthesis. The central prenyl (4*R*)-[4-^{3}H]-label is retained in the biosynthesis and is transferred to C-2 of the trichothecane. The relative numbers of [2-$^3H_2$]- and [5-$^3H_2$]-mevalonoid labels that are incorporated suggest that the carbonyl group of trichothecin might arise through the rearrangement of an epoxide.

THE trichothecane antibiotics are a group of sesquiterpenoid fungal metabolites possessing the 12,13-epoxytrichothec-9-ene (1)[2] nucleus. They possess a diverse range of additional oxygen-containing substituents, the commonest of which is a C-4 hydroxy-group or its derivative. They are produced by a number of micro-organisms, including *Trichothecium roseum* and various *Fusaria*,



OPP = pyrophosphate

Myrothecia, and *Trichoderma* species.[3] Several of the naturally occurring esters of the 12,13-epoxytrichothec-9-ene alcohols show cytotoxic, antifungal, or phytotoxic activity.[4] Biogenetically these substances may be derived from farnesyl pyrophosphate by two 1,2-methyl shifts.[5] There are, however, two ways of coiling this chain [(2) and (3)] to afford (on cyclisation) the trichothecane skeleton. Most biogenetic proposals have implicated γ-bisabolene (4) as an intermediate in the cyclisation.

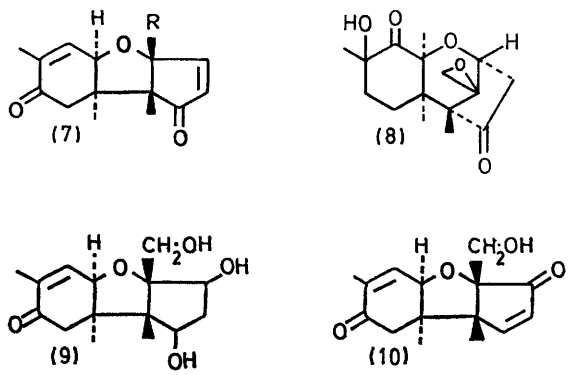
We have approached the problem in two ways. First, the position of the mevalonoid hydrogen atoms in the acyclic farnesyl pyrophosphate is known from work on steroid biosynthesis.[6] By feeding experiments with doubly labelled mevalonates of known ^{3}H : ^{14}C ratio, in which the ratio in the metabolites and their degradation products is then determined, it is possible to determine the number and location of the mevalonoid hydrogen atoms that are incorporated and thus to obtain information on post-farnesyl pyrophosphate stages. Second, by employing specifically labelled geranyl and farnesyl pyrophosphates, it is possible to find out from which prenyl unit (distal, central, or terminal) a particular hydrogen atom originates. Not only does this serve to confirm deductions from the mevalonate pattern but it may also reveal unsuspected hydride shifts. Combining these two approaches, we have sought information on the manner of folding and cyclisation of the farnesol chain and on the rearrangement stages.

Two of the less-highly oxygenated trichothecanes are trichodermol (5)[7] (roridin C[8]) and trichothecin (6),[9] which are produced by *Trichoderma sporulosum* and *Trichothecium roseum*, respectively. Previous work[5] has has shown that trichothecin incorporates 3 mol. equiv. of [2-^{14}C]mevalonic acid and has established a labelling pattern. It has also been shown[10] that farnesyl pyrophosphate is a precursor of trichothecin. Parallel work[11] on verrucarol is in substantial agreement with the results reported here, some of which have formed the subject of preliminary communications.[12] [2-^{3}H,2-^{14}C]-, (4*R*)-[4-^{3}H,2-^{14}C]-, and [5-$^3H_2$,2-^{14}C]-mevalonic acids were fed to *Trichoderma sporulosum* and to *Trichothecium*

[1] Part VII, Miss P. M. Adams and J. R. Hanson, *J.C.S. Perkin I*, 1972, 586.
[2] W. O. Godtfredsen, J. F. Grove, and C. Tamm, *Helv. Chim. Acta*, 1967, **50**, 1666.
[3] For a review see W. B. Turner, 'Fungal Metabolites,' Academic Press, London, 1971, p. 219.
[4] J. F. Grove and P. H. Mortimer, *Biochem. Pharmacol.*, 1969, **18**, 1473.
[5] E. R. H. Jones and G. Lowe, *J. Chem. Soc.*, 1960, 3959; for a review see W. Parker, J. S. Roberts, and R. Ramage, *Quart. Rev.*, 1967, **21**, 331.
[6] G. Popjak and J. W. Cornforth, *Biochem. J.*, 1966, **101**, 553.
[7] W. O. Godtfredsen and S. Vangedal, *Acta Chem. Scand.*, 1965, **19**, 1088.
[8] B. Bohner, E. Fetz, E. Harri, H. P. Sigg, C. Stoll, and C. Tamm, *Helv. Chim. Acta*, 1965, **48**, 1079.
[9] G. G. Freeman and R. I. Morrison, *Nature*, 1948, **162**, 30; G. G. Freeman, J. E. Gill, and W. S. Waring, *J. Chem. Soc.*, 1959, 1105; J. Fishman, E. R. H. Jones, G. Lowe, and M. C. Whiting, *ibid.*, 1960, 3948.
[10] B. Achilladelis and J. R. Hanson, *Phytochemistry*, 1968, **7**, 589.
[11] R. Achini, B. Muller, and C. Tamm, *Chem. Comm.*, 1971, 404.
[12] B. Achilladelis, P. M. Adams, and J. R. Hanson, *Chem. Comm.*, 1970, 511; P. M. Adams and J. R. Hanson, *ibid.*, p. 1569.

roseum. The metabolites were isolated and the esters were hydrolysed to their more readily purified, higher melting, parent alcohols. The results are shown in the Table.

The metabolites were degraded as follows. The trichodermol from the [2-^{3}H,2-^{14}C]mevalonate feed was oxidised to the 4-ketone, trichodermone,[7] ^{3}H : ^{14}C ratio 6·06 : 1, corresponding to the loss of one tritium label from C-4. The trichodermol was converted into its 9,10-epoxide, ^{3}H : ^{14}C ratio 7·32 : 1. Trichothecolone, on oxidation to the C-4 ketone, trichothecodione, ^{3}H : ^{14}C ratio 2·64 : 1, lost 2·04 labels. The trichothecodione was rearranged[9] to neotrichothecodione (7; R = CH_2·OH), ^{3}H : ^{14}C ratio 2·7 : 1. The primary alcohol system corresponding to the epoxide grouping of trichothecolone was then oxidised to a carboxylic acid (7; R = CO_2H), ^{3}H : ^{14}C ratio 2·68 : 1. There was thus no loss of label from C-13.



The trichodermol from the (4*R*)-[4-^{3}H,2-^{14}C]mevalonate feed was oxidised to trichodermone, ^{3}H : ^{14}C ratio 5·85 : 1, and converted with osmium tetroxide in pyridine into its 9,10-glycol, ^{3}H : ^{14}C ratio 6·03 : 1. This glycol was oxidised to a ketol (8), ^{3}H : ^{14}C ratio 2·87 : 1, thus locating one tritium label at C-10. The second tritium label from (4*R*)-[4-^{3}H,2-^{14}C]mevalonate was located by using trichothecolone. This was converted into trichothecolone glycol (9) and the latter was oxidised to the αβ-unsaturated ketone (10), ^{3}H : ^{14}C ratio 4·7 : 1, with the loss of one label. Consequently a 4-*pro*-*R*-mevalonoid hydrogen atom was located at C-2.

The trichothecin from the [5-^{3}H,2-^{14}C]mevalonate feed was hydrolysed to trichothecolone, ^{3}H : ^{14}C ratio 4·0 : 1, with the loss of two tritium labels. The trichothecolone was oxidised to the C-4 ketone, trichothecodione, ^{3}H : ^{14}C ratio 4·0 : 1, and the latter was converted into neotrichothecodione (7; R = CH_2·OH), ^{3}H : ^{14}C ratio 1·2 : 1, with the loss of 1·6 labels. Consequently two [5-$^3H_2$]-mevalonoid labels are distributed between the three enolic protons (at C-7 and C-11) on ring A and two on ring C at C-2, the latter becoming transiently enolic during the formation of neotrichothecodione.

The problem then remained to determine whether these mevalonoid hydrogen atoms originated from the distal, central, or terminal prenyl units of farnesyl pyrophosphate. [1-$^3H_2$,2-^{14}C]Farnesyl pyrophosphate (^{3}H : ^{14}C ratio 37·7 : 1) was fed to *Trichothecium roseum* and the trichothecolone was isolated. This showed a ^{3}H : ^{14}C ratio of 20·8 : 1; hence one label had been lost. Similarly [1-$^3H_2$,2-^{14}C]farnesyl pyrophosphate (^{3}H : ^{14}C ratio 32·0 : 1) was fed to *Trichoderma sporulosum*. The trichodermol, ^{3}H : ^{14}C ratio 19·4 : 1, was isolated and oxidised to trichodermone, ^{3}H : ^{14}C ratio 16·3 : 1. [2-^{3}H,2-^{14}C]Farnesyl pyrophosphate (^{3}H : ^{14}C ratio 7·4 : 1) was fed to *Trichothecium roseum* and the trichothecolone was isolated. This showed a ^{3}H : ^{14}C ratio of 7·0 : 1; thus no label was lost on cyclisation. [2-^{3}H,2-^{14}C]Geranyl pyrophosphate (^{3}H : ^{14}C ratio 14·0 : 1) was fed to *Trichothecium roseum*. The trichothecin isolated showed a ^{3}H : ^{14}C ratio of 13·7 : 1. This was hydrolysed to trichothecolone, which showed a ^{3}H : ^{14}C ratio of 15·3 : 1. Another sample of the pyrophosphate (^{3}H : ^{14}C ratio 6·5 : 1) was fed to *Trichoderma sporulosum*, and trichodermol of ^{3}H : ^{14}C ratio 5·45 : 1 was isolated. This showed that the 4-*pro*-*R*-hydrogen atom from the central prenyl unit was retained in the biosynthesis.

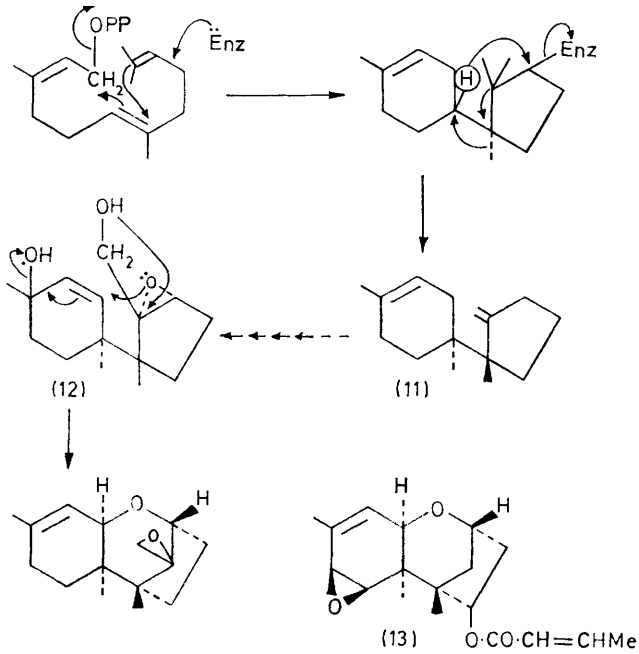
Incorporation of mevalonoid labels into trichodermol and trichothecolone

	[2-$^3H_2$]Mevalonate			(4*R*)-[4-^{3}H]Mevalonate			[5-$^3H_2$]Mevalonate		
	^{3}H : ^{14}C ratio	No. of labels	Incorp. (%)	^{3}H : ^{14}C ratio	No. of labels	Incorp. (%)	^{3}H : ^{14}C ratio	No. of labels	Incorp. (%)
Mevalonic acid	9·2 : 1	6		8·68 : 1	3				
Trichodermol	7·5 : 1	4·9	0·23	5·83 : 1	2	6·9			
Mevalonic acid	7·99 : 1	6		13·4 : 1	3		11·6 : 1	6	
Trichothecolone	5·4 : 1	4·02	0·9	8·7 : 1	2	1·6	7·2 : 1	3·8	0·8 *
Mevalonic acid	3·97 : 1	6							
Trichothecin	2·60 : 1	3·93	0·24						

* As trichothecin.

The following conclusions may be drawn. First, the (4*R*)-[4-^{3}H]mevalonate labelling pattern, the number of [2-$^3H_2$]mevalonate labels incorporated into trichodermol, and the number of labels incorporated from the farnesyl pyrophosphate indicate that the chain is folded in manner (2) rather than (3). This conclusion, which is drawn from tritium-labelling experiments and is in agreement with other work,[11] differs from the earlier results based on carbon-14 experiments.[5] We are unable to provide a satisfactory explanation for this discrepancy. Second, the absence of a [2-^{3}H]mevalonoid label on the epoxide grouping demonstrates, in confirmation of the earlier experiments, that the methyl groups of the starter unit

in the farnesyl pyrophosphate precursor of the trichothecanes retain their individuality. This is in contrast to recent results with the picrotoxane sesquiterpenes.[13] Third, the incorporation of the [2-^{3}H]geranyl pyrophosphate indicates that the central 4-*pro*-*R*-mevalonoid hydrogen atom is retained during the biosynthesis and that it migrates to C-2 in the trichothecane metabolites. This excludes a γ-bisabolene intermediate (4) from the biosynthesis. In view of the recent isolation of trichodiene (11) and trichodiol (12)[14] from *Trichothecium roseum*, this leads to the illustrated proposal (see Scheme) for the cyclisation sequence to form the 12,13-epoxytrichothec-9-ene skeleton. Fourth, the trichothecolone isolated contained four [2-^{3}H]mevalonoid



SCHEME

labels and trichodermol five. If the carbonyl group of trichothecolone was formed in a simple oxidative step then trichothecolone would be expected to contain three [2-^{3}H]mevalonoid labels. On the other hand trichothecin isolated contained only four of the expected five [5-$^3H_2$]mevalonoid labels. In both these cases one label is lost in an easy exchange reaction, and we suggest that these are being lost from C-7. Trichothecolone in deuterium oxide readily incorporates two deuterium atoms to give material showing an $M + 2$ peak at 264 in the mass spectrum. These results suggest that a label from the terminal mevalonate unit migrates from C-8 to C-7 in trichothecin. The epoxide crotocin (13),[15] which co-occurs [16] with trichothecin, or a similar epoxide, may be a precursor of trichothecin, with an NIH type of shift occurring in the isomerisation of the epoxide to the ketone.

EXPERIMENTAL

General experimental details have been described previously.[1,10]

[2-$^3H_2$,2-^{14}C]*Mevalonic Acid Feeds.*—(i) *To* Trichoderma sporulosum. The mevalonate (^{3}H : ^{14}C 9·2 : 1) in ethanol (30 ml) was added to 3 l of *Trichoderma sporulosum* 6 days after inoculation. After 92 h the metabolites were isolated. The crude extract was adsorbed on silica gel and chromatographed on alumina. Elution with 10—12·5% ethyl acetate–light petroleum gave trichodermin (0·55 g). The trichodermin (0·5 g) in ethanol (5·0 ml) was heated under reflux with aqueous 2N-sodium hydroxide for 90 min. The ethanol was removed *in vacuo* and the trichodermol was recovered in ether. Recrystallisation from light petroleum gave needles (0·32 g), m.p. 116—119° (lit.,[7] 117·5—118°) (^{3}H : ^{14}C 7·51 : 1).

Oxidation of the trichodermol. Trichodermol (45 mg) in acetic acid (0·1 ml) was treated with 10% chromium trioxide in 95% acetic acid (0·3 ml). After 15 min, water (1 ml) was added; the trichodermone was filtered off and crystallised from ether as needles (16 mg), m.p. 152—155° (lit.,[7] 154—155°) (^{3}H : ^{14}C 6·06 : 1). 9,10:12,13-Diepoxytrichothecan-4-ol,[17] prepared from trichodermol and *m*-chloroperbenzoic acid, had m.p. 209—211° (^{3}H : ^{14}C 7·32 : 1).

(ii) *To* Trichothecium roseum. (*a*) The mevalonate (^{3}H : ^{14}C 7·99 : 1) in ethanol (10 ml) was added to 3 l of *Trichothecium roseum* 14 days after inoculation. After a further 7 days the ferment was extracted with ethyl acetate and the trichothecolone (250 mg), m.p. 176—178° (^{3}H : ^{14}C 5·4 : 1), isolated by chromatography on alumina in 30—50% ethyl acetate–light petroleum.

(*b*) The *NN'*-dibenzylethylenediammonium salt of the mevalonate (^{3}H : ^{14}C 6·6 : 1) in water (30 ml) was added to 3 l of *Trichothecium roseum* 14 days after inoculation. After a further 7 days the ferment was extracted with ethyl acetate to afford a gum (1·57 g), which was chromatographed on alumina. Elution with increasing concentrations of ethyl acetate in light petroleum gave trichothecin (50 mg), m.p. 116—118° (^{3}H : ^{14}C 4·3 : 1), and trichothecolone (500 mg), m.p. 176—178° (^{3}H : ^{14}C 4·1 : 1).

Oxidation of trichothecolone. Trichothecolone (400 mg) (^{3}H : ^{14}C 5·4 : 1) in acetone (4 ml) was treated with 8N-chromium trioxide reagent (0·4 ml) for 0·5 h. The solution was diluted with water and the trichothecodione was filtered off; it crystallised from acetone–light petroleum as needles, m.p. 218—219° (lit.,[9] 218—219°) (^{3}H : ^{14}C 2·64 : 1).

Neotrichothecodione. Trichothecodione (200 mg) in methanol (7 ml) was treated at 50° with sodium carbonate (200 mg) in water (7 ml) to yield neotrichothecodione (136 mg), which crystallised from benzene–light petroleum as needles, m.p. 165—166° (lit.,[9] 168—169°) (^{3}H : ^{14}C 2·7 : 1).

Oxidation to the carboxylic acid (7; R = CO_2H). Neotrichothecodione (45 mg) in acetone (1 ml) was treated with an excess of 8N-chromium trioxide reagent. After 1 h the excess of reagent was destroyed with methanol, the solution was diluted with water, and the product was recovered in chloroform. The acid crystallised from benzene–light

[13] M. Bioilaz and D. Arigoni, *Chem. Comm.*, 1969, 633; A. Corbella, P. Gariboldi, G. Jommi, and C. Scholastico, *ibid.*, 1969, 634.
[14] S. Nozoe and Y. Machida, *Tetrahedron Letters*, 1970, 1177, 2671.
[15] J. Gyimesi and A. Melera, *Tetrahedron Letters*, 1967, 1665.
[16] B. Achilladelis and J. R. Hanson, *Phytochemistry*, 1969, **8**, 765.
[17] P. M. Adams and J. R. Hanson, unpublished work.

petroleum as needles, m.p. 112—114° (lit.,[9] 115—116°) (^{3}H : ^{14}C 2·68 : 1).

(4R)-[4-^{3}H,2-^{14}C]*Mevalonic Acid Feeds.*—(i) *To* Trichodermasporulosum. The mevalonate (^{3}H : ^{14}C 8·68 : 1) in ethanol (27 ml) added to 2·7 l of *Trichoderma sporulosum* 6 days after inoculation. The ferment was harvested after a further 10 days and the metabolites were recovered in ethyl acetate. The crude trichodermin (3·49 g) in ethanol (50 ml) was heated under reflux with aqueous 2N-sodium hydroxide (15 ml) for 90 min. The ethanol was removed under reduced pressure and the trichodermol (2·28 g) was recovered in ether. It crystallised from light petroleum as needles, m.p. 117—118° (lit.,[7] 117·5—118°) (^{3}H : ^{14}C 5·83 : 1).

Degradation of the trichodermol. The trichodermol (1 g) was oxidised to trichodermone (0·48 g), m.p. 154—156° (^{3}H : ^{14}C 5·82 : 1), as described previously. The trichodermone (0·47 g) in pyridine (5 ml) was treated with osmium tetroxide (500 mg) in pyridine (5 ml) at room temperature for 48 h. Sodium hydrogen sulphite (2 g) in water (25 ml) and pyridine (5 ml) was added, and the mixture was stirred for 4 h, poured into water, and acidified with dilute hydrochloric acid. The glycol [17] (0·30 g), m.p. 210—211° (^{3}H : ^{14}C 6·03 : 1), was recovered in ethyl acetate. The glycol (200 mg) in acetone (4 ml) was treated with 8N-chromium trioxide reagent (0·4 ml) for 1 h. Methanol was added, the solvent was evaporated off, the solution was diluted with water, and the product was recovered in ethyl acetate and purified by preparative layer chromatography in 50% ethyl acetate–light petroleum. The ketol [17] (8) (70 mg) crystallised from light petroleum as needles, m.p. 162—166° (^{3}H : ^{14}C 2·87 : 1).

(ii) *To* Trichothecium roseum. The mevalonate (^{3}H : ^{14}C 13·4 : 1) was added to 3 l of *Trichothecium roseum* 21 days after inoculation. The ferment was harvested after a further 7 days and the trichothecolone, m.p. 183° (lit.,[9] 183—184°) (^{3}H : ^{14}C 8·7 : 1), was recovered as described previously.

Degradation of the trichothecolone. Trichothecolone (300 mg) was heated under reflux with 0·5N-sulphuric acid (25 ml) for 2 h. The solution was neutralised with aqueous sodium hydrogen carbonate and the trichothecolone glycol (180 mg) was filtered off; it crystallised from acetone–light petroleum as needles, m.p. 192—194° (lit.,[9] 193—194°). The glycol (150 mg) was treated with aqueous 5% chromium trioxide (5 ml) for 1 h and the product was then filtered. The αβ-unsaturated ketone (10) (60 mg) crystallised from acetone–light petroleum as needles, m.p. 228—229° (lit.,[9] 228—229°) (^{3}H : ^{14}C 4·7 : 1).

[5-^{3}H$_2$,2-^{14}C]*Mevalonic Acid Feed.*—The mevalonate (^{3}H : ^{14}C 11·6 : 1) in ethanol (30 ml) was added to 3 l *Trichothecium roseum* 12 days after inoculation. The ferment was harvested after a further 10 days. The trichothecin (300 mg) was isolated as described previously. It crystallised from light petroleum as needles, m.p. 117—118° (lit.,[9] 118°) (^{3}H : ^{14}C 7·2 : 1). On hydrolysis with ethanolic N-potassium hydroxide this afforded trichothecolone, m.p. 181° (lit.,[9] 183—184°) (^{3}H : ^{14}C 4·0 : 1).

Degradation of the trichothecolone. Trichothecolone (85 mg) in acetone (4 ml) was treated with 8N-chromium trioxide reagent (0·15 ml) for 1 h. The solution was diluted with water to afford trichothecodione (65 mg), which crystallised from acetone–light petroleum as needles, m.p. 218° (lit.,[9] 218—219°) (^{3}H : ^{14}C 4·0 : 1). The trichothecodione (50 mg) was heated at 60° with sodium carbonate (100 mg) in aqueous methanol (1 : 1; 2 ml) for 1 h to afford neotrichothecodione (7; R = CH_2·OH), m.p. 165° (lit.,[9] 165—166°) (^{3}H : ^{14}C 1·2 : 1).

Farnesyl Pyrophosphate Feeds.—(*a*) [1-^{3}H$_2$,2-^{14}C]Farnesyl pyrophosphate (^{3}H : ^{14}C 37·66 : 1) was added to 2·8 l of *Trichothecium roseum* 9 days after inoculation. The ferment was harvested after a further 17 days and the trichothecolone (0·46 g), m.p. 183—185° (lit.,[9] 183—184°) (^{3}H : ^{14}C 20·3 : 1), was isolated as described previously.

(*b*) [1-^{3}H$_2$,2-^{14}C]Farnesyl pyrophosphate (^{3}H : ^{14}C 32·0 : 1) in aqueous ethanol (30 ml) was fed to 3 l of *Trichoderma sporulosum* 7 days after inoculation. The ferment was harvested after a further 7 days and the crude gum (0·8 g) was hydrolysed to give trichodermol (0·25 g), m.p. 117—118° (^{3}H : ^{14}C 19·1 : 1). The trichodermol (200 mg) was oxidised with 8N-chromium trioxide reagent as described previously to give trichodermone (113 mg), m.p. 153—155° (^{3}H : ^{14}C 16·3 : 1).

(*c*) [2-^{3}H,2-^{14}C]Farnesyl pyrophosphate (^{3}H : ^{14}C 7·4 : 1) was added to 2 l of *Trichothecium roseum* 10 days after inoculation. The ferment was harvested after a further 8 days and the trichothecolone (0·30 g), m.p. 184—185° (^{3}H : ^{14}C 7·0 : 1), was isolated as described previously.

[2-^{3}H,2-^{14}C]*Geranyl Pyrophosphate Feeds.*—(*a*) The pyrophosphate (^{3}H : ^{14}C 14·0 : 1) in aqueous ethanol (3 ml) was added to 5 l of *Trichothecium roseum* 15 days after inoculation. After a further 6 days the ferment was harvested and the trichothecin (300 mg), m.p. 115—117° (^{3}H : ^{14}C 13·7 : 1) was isolated. The trichothecin (200 mg) was converted into trichothecolone (121 mg), m.p. 182—183° (^{3}H : ^{14}C 15·3 : 1) with methanolic potassium hydroxide.

(*b*) The pyrophosphate (^{3}H : ^{14}C 6·5 : 1) in aqueous ethanol (16 ml) was added to 1·6 l of *Trichoderma sporulosum* 8 days after inoculation. The ferment was harvested after a further 5 days. The crude extract (0·27 g) was hydrolysed with ethanolic 2N-sodium hydroxide and the product purified by preparative layer chromatography in 40% ethyl acetate–light petroleum to afford trichodermol (15 mg), m.p. 114—116° (^{3}H : ^{14}C 5·45 : 1).

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