Studies in Terpenoid Biosynthesis. Part XII.[1] Carbon-13 Nuclear Magnetic Resonance Spectra of the Trichothecanes and the Biosynthesis of Trichothecolone from [2-^{13}C]Mevalonic Acid

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The ^{13}C n.m.r. signals of the trichothecanes have been assigned. [2-^{13}C]Mevalonic acid has been prepared and incorporated into trichothecolone. C-4, C-14, and C-8 were enriched indicating that farnesyl pyrophosphate is folded in the manner (3).

FARNESYL PYROPHOSPHATE is a precursor of the sesquiterpenoid fungal metabolite, trichothecolone (1).[2] In their study [3] of the biosynthesis of trichothecin (4) Jones and Lowe degraded material derived from [2-^{14}C]mevalonic acid to show that C-10 was labelled, from which they concluded that a farnesyl pyrophosphate precursor would be folded in the manner (2) prior to cyclization. In our more recent biosynthetic work [4] we showed that C-10 bore a 4-*pro*-*R*-^{3}H-mevalonoid label. This implied either that a hydrogen migration had occurred during the biosynthesis or that the farnesyl pyrophosphate was folded in the manner (3) contrary to the earlier results. In this paper we report the assignment of the ^{13}C n.m.r. spectra of a series of trichothecanes, the incorporation of [2-^{13}C]mevalonic acid into trichothecolone by *Trichothecium roseum*, and the location of the enriched atoms. During the course of this work Nozoe and Machida,[5] independently repeating our [4-*pro*-*R*-^{3}H,2-^{14}C]mevalonate experiment, in a seven step chemical degradation showed that C-8 was labelled by [2-^{14}C]mevalonate. The

[1] Part XI, J. R. Hanson and T. Marten, *J.C.S. Perkin I*, 1974, 857.
[2] B. Achilladelis and J. R. Hanson, *Chem. and Ind.*, 1967, 1643; *Phytochemistry*, 1969, **8**, 765.
[3] E. R. H. Jones and G. Lowe, *J. Chem. Soc.*, 1960, 3959.
[4] B. Achilladelis, P. M. Adams, and J. R. Hanson, *Chem. Comm.*, 1970, 511; *J.C.S. Perkin I*, 1972, 1425.
[5] S. Nozoe and Y. Machida, *Tetrahedron*, 1972, **28**, 5105.

incorporation of [2-^{13}C]mevalonate into the related fungal metabolite, helicobasidin, has very recently been described.[6]

The trichothecane group of fungal metabolites is increasing in number.[7] The characteristic ^{1}H n.m.r. spectra have played [8] a significant role in their structural determination to which an analysis of their ^{13}C n.m.r. could now play an additional role. The ^{13}C n.m.r. spectra of the trichothecanes [(1) and (4)—(11)] which possess a variety of substituents on rings A and C and at C-15, were determined. The spectra were obtained at 25·15 MHz using a pulsed Fourier transform system with proton noise decoupling, off-resonance decoupling, and in one instance, single proton resonance decoupling. The results are in Table 1. The resonances were assigned in the following manner.

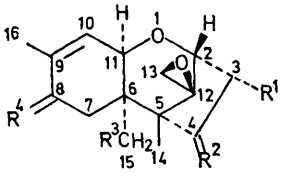
TABLE 1

^{13}C N.m.r. spectra of the trichothecanes (δ in p.p.m. from Me_4Si)

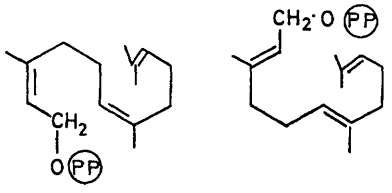
	Carbon atom							
	2	3	4	5	6	7	8	9
Trichothecin [a] (4) [9]	70·1 (d)	36·95 (t)	73·35 (d)	49·0 (s)	43·7 (s)	42·1 (t)	198·5 (s)	138·0 (s)
Trichothecolone (1) [9]	70·2 (d)	40·2 (t)	73·1 (d)	49·5 (s)	43·6 (s)	42·35 (t)	199·4 (s)	138·5 (s)
Trichodermol (5) [10]	70·8 (d)	40·2 (t)	74·2 (d)	49·6 (s)	40·4 (s)	24·85 (t)	28·4 (t)	140·5 (s)
Epitrichodermol (6) [10]	70·3 (d)	36·4 (t)	78·0 (d)	47·0 (s)	40·4 (s)	25·5 (t)	27·8 (t)	139·7 (s)
Trichodermone (7) [10]	71·4 (d)	42·0 (t)	214·9 (s)	55·3 (s)	42·7 (s)	23·9 (t)	27·9 (t)	140·9 (s)
Calonectrin [b] (8) [7]	68·2 (d)	71·3 (d)	39·4 (t)	45·4 (s)	43·0 (s)	21·05 (t)	28·2 (t)	140·3 (s)
3-Deacetylcalonectrin [c] (9) [7]	68·25 (d)	68·8 (d)	42·2 (t)	45·75 (s)	43·0 (s)	21·05 (t)	28·3 (t)	140·15 (s)
Diacetoxyscripenol [d] (10) [11]	67·9 (d)	83·9 (d)	77·6 (d)	48·9 (s)	44·0 (s)	20·9 (t)	27·9 (t)	140·15 (s)
Triacetoxyscirpenol [e] (11) [11]	67·95 (d)	78·4 (d)	77·5 (d)	48·8 (s)	44·05 (s)	20·9 (t)	27·85 (t)	140·5 (s)

	10	11	12	13	14	15	16
Trichothecin [a] (4) [9]	137·2 (d)	79·5 (d)	65·5 (s)	47·4 (t)	5·7 (q)	15·4 (q)	18·4 (q)
Trichothecolone (1) [9]	137·7 (d)	79·6 (d)	66·00 (s)	47·3 (t)	6·2 (q)	15·6 (q)	18·5 (q)
Trichodermol (5) [10]	119·4 (d)	79·3 (d)	66·2 (s)	47·9 (t)	6·4 (q)	16·0 (q)	23·4 (q)
Epitrichodermol (6) [10]	119·5 (d)	80·2 (d)	66·25 (s)	49·1 (t)	9·9 (q)	15·7 (q)	23·3 (q)
Trichodermone (7) [10]	118·7 (d)	76·25 (d)	65·3 (s)	50·1 (t)	5·4 (q)	15·4 (q)	23·4 (q)
Calonectrin [b] (8) [7]	119·0 (d)	78·1 (d)	65·0 (s)	48·5 (t)	12·2 (q)	63·7 (t)	23·2 (q)
3-Deacetylcalonectrin [c] (9) [7]	119·1 (d)	79·9 (d)	65·5 (s)	48·4 (t)	12·3 (q)	63·7 (t)	23·2 (q)
Diacetoxyscirpenol [d] (10) [11]	118·7 (d)	79·7 (d)	64·4 (s)	47·0 (t)	6·7 (q)	63·5 (t)	23·2 (q)
Triacetoxyscirpenol [e] (11) [11]	118·4 (d)	79·3 (d)	64·1 (s)	47·1 (t)	6·55 (q)	63·5 (t)	23·2 (q)

[a] C-1′, 166·1 (s); C-2′ 120·4 (d); C-3′ 145·9 (d); C-4′ 15·4 (q). [b] Two acetate methyls: 21·17 (q); two acetate carbonyls 170·6 (s). [c] Acetate methyl: 21·05 (q); acetate carbonyl: 170·7 (s). [d] Two acetate methyls: 20·1 (q); acetate carbonyls: 170·05 (s), 171·7 (s). [e] Two acetate methyls at 20·8 (q), one acetate methyl at 21·3 (q); one acetate carbonyl at 169·7 (s), two acetate carbonyls at 170·4 (s).



(1) R^1 = H, R^2 = β-OH,α-H, R^3 = H, R^4 = O
(4) R^1 = H, R^2 = β-O·CO·CH=CH·CH_3,α-H, R^3 = H, R^4 = O
(5) R^1 = H, R^2 = β-OH,α-H, R^3 = H, R^4 = H_2
(6) R^1 = H, R^2 = α-OH,β-H, R^3 = H, R^4 = H_2
(7) R^1 = H, R^2 = O, R^3 = H, R^4 = H_2
(8) R^1 = OAc, R^2 = H_2, R^3 = OAc, R^4 = H_2
(9) R^1 = OH, R^2 = H_2, R^3 = OAc, R^4 = H_2
(10) R^1 = OH, R^2 = β-OAc,α-H, R^3 = OAc, R^4 = H_2
(11) R^1 = OAc, R^2 = β-OAc,α-H, R^3 = OAc, R^4 = H_2



(2) (3)

C-2, C-3, *and* C-4.—All the substances showed a doublet in the off-resonance spectra at approximately 70 p.p.m. which was assigned to C-2 whilst the C-4 alcohols showed a doublet at 74 p.p.m. which was absent in trichodermone and was therefore assigned to C-4. This resonance showed a change of 4 p.p.m. on epimerization of trichodermol at C-4. The distinction between the C-2, C-3, and C-4 resonances in compounds (8)—(11) was made on the basis of the spectral changes on acetylation.[12] Thus calonectrin (8) exhibited doublets at 68 and 71 p.p.m. whilst the alcohol (9) had similar resonances at 68 and 69 p.p.m. and hence the lower field resonance was assigned to C-3. However, the C-3 resonance showed an upfield shift (83·9 to 78·4 p.p.m.) on acetylation of diacetoxyscirpenol [(10) ⟶ (11)]. An oxygen substituent at C-3 causes an upfield shift in the C-2 resonance. A triplet at 40 p.p.m. which was present in the compounds without substituents at C-3, was assigned to C-3.

[6] M. Tanabe, K. T. Suzuki, and W. C. Jankowski, *Tetrahedron Letters*, 1973, 4723.

[7] (*a*) For a literature survey see W. B. Turner, 'Fungal Metabolites,' Academic Press, London, 1971; (*b*) J. F. Grove, *J. Chem. Soc.* (*C*), 1970, 375, 378; (*c*) D. Gardner, A. T. Glen, and W. B. Turner, *J.C.S. Perkin I*, 1972, 2576.

[8] J. Gutzwiller, R. Mauli, H. P. Sigg, and Ch. Tamm, *Helv. Chim. Acta*, 1964, **47**, 2234; B. K. Tidd, *J. Chem. Soc.* (*C*), 1967, 218.

[9] G. G. Freeman, J. E. Gill, and W. S. Waring, *J. Chem. Soc.*, 1959, 1105.

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[12] *Cf.* H. J. Reich, M. Jautelat, M. T. Messe, F. J. Weigert, and J. D. Roberts, *J. Amer. Chem. Soc.*, 1969, **91**, 7445.

C-13.—All the compounds showed a triplet in the off-resonance spectrum at approximately 48 p.p.m. which was assigned to C-13. Furthermore when trichothecin (4) was irradiated at τ 7·0 in the proton spectrum at the centre of the AB quartet due to the C-13 protons,[8] this signal appeared as a singlet.

C-5 *and* C-6.—These two fully substituted carbon atoms exhibited singlets between 40 and 55 p.p.m. in the off-resonance spectra. The resonances were differentiated by the effects of adjacent substituents. For example the lower field signal in trichodermone (7) at 55·3 p.p.m. reflects the downfield shift due to an adjacent carbonyl group.[13] Conversely the absence of a C-4 substituent, as in calonectrin (8), leads to a signal at 45·4 p.p.m. A comparison of trichothecolone (1) and trichothecin (4) with trichodermol (5) and epitrichodermol (6) showed a downfield shift (*ca.* 3 p.p.m.) in the C-6 resonance owing to the presence of the carbonyl group at C-8, an effect which is also produced by an α-oriented C-3 substituent.

C-7 *and* C-8.—The compounds lacking a C-8 carbonyl group show two triplets between 20 and 28 p.p.m. of which the lower field resonance was assigned to C-8 owing to the effect of the 9,10-double bond. In trichothecin (4) and trichothecolone (1) the C-8 carbonyl group could be readily assigned as the resonance at lowest field in the spectrum. The carbonyl group also caused a large downfield shift in the adjacent C-7 methylene resonance (24 to 42 p.p.m.).[13] Substitution at C-15 caused a small upfield shift in this resonance.

C-9 *and* C-10.—The olefinic carbon resonances were assigned by analogy with 2-methylcyclohex-2-enone [14] and by off-resonance experiments in which the C-9 resonance remained a singlet whilst C-10 became a doublet.

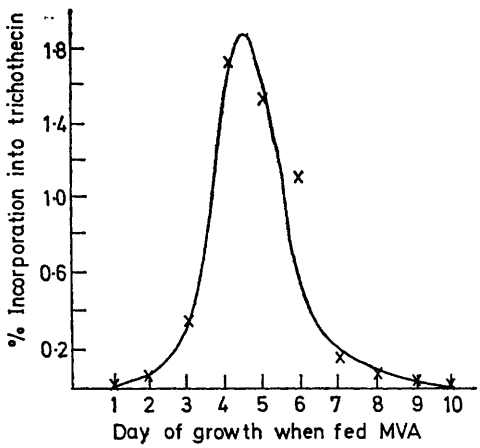
C-11 *and* C-12.—A doublet at 79 and a singlet at 65 p.p.m. in the off-resonance spectra were assigned to C-11 and C-12 since these showed little variation with structural changes. A small upfield shift for the C-12 resonance in trichodermone (7) may reflect the influence of the shielding cone of the carbonyl group.

C-14, C-15, *and* C-16.—The methyl group resonances appeared as quartets in the off-resonance spectra. A C-4 oxygen substituent produces a marked shielding of C-14 [*cf.* (9) and (10)], an effect which can also be seen in the steroid series between 17β-substituents and the C-18 methyl group.[15] The olefinic methyl resonance (C-16) showed a characteristic upfield shift on the introduction of a carbonyl group at C-8.

The side chain resonances of trichothecin (4) were assigned by analogy with crotonaldehyde [13] whilst the acetate carbonyl and methyl resonances appeared at the expected positions.

Having assigned the relevant trichothecane resonances, the biosynthetically enriched material was then prepared. [2-^{13}C]Mevalonic acid was prepared from [2-^{13}C]-acetic acid (91·5% enriched) *via* methyl bromoacetate and a Reformatski reaction with 4-chlorobutan-2-one.[16] The success of this latter reaction depended on the activation of 40 mesh zinc with dilute hydrochloric acid immediately prior to use and on careful control of the reaction temperature at 40°. Higher reaction temperatures led to dehydration and dehydrochlorination. The methyl 5-chloro-3-hydroxy-3-methylpentanoic acid was carefully distilled below 50° *in vacuo* and then hydrolysed first with alkali in dimethylformamide and then with acid to afford [2-^{13}C]mevalonic acid lactone in 24% overall yield.

Some trial experiments were carried out to optimize the incorporation of mevalonate into trichothecin by *Trichothecium roseum.* In a time *versus* incorporation experiment, the incorporation of [2-^{14}C]mevalonate into trichothecin over a 24 h period showed a sharp maximum between days 4 and 5 after innoculation (see Figure).



In a mass *versus* incorporation experiment, the incorporation appeared to decrease above 100 mg l^{-1} of mevalonate added to the broth. The [2-^{13}C]mevalonic acid, containing a small amount of [2-^{14}C]mevalonic acid as a tracer, was then added at a concentration of 100 mg l^{-1} to a three day old culture of *Trichothecium roseum.* This was then harvested after a further 5 days growth to afford trichothecin. The crude trichothecin was hydrolysed to trichothecolone (1) which is more easily purified. The incorporation at each centre based on [2-^{14}C]mevalonate, was 0·3%. The noise decoupled ^{13}C n.m.r. spectra of the enriched and unenriched material were then compared. The peak heights were normalized onto that of C-12, a tertiary centre which was one of the least likely to have been derived from [2-^{13}C]mevalonate or to be affected by scrambling; on current biogenetic theory it would be derived from a carboxy-group of acetate. Based on a natural abundance of 1·1%, C-14 showed an additional 0·36% incorporation of ^{13}C, C-4 a 0·35% incorporation, and C-8 a 0·35% incorporation. These were the only centres to show any enrichment.

[13] *Cf.* H. Eggert and C. Djerassi, *J. Org. Chem.*, 1973, **38**, 3788.
[14] D. H. Marr and J. B. Stothers, *Canad. J. Chem.*, 1965, **43**, 596.
[15] T. A. Wittstruck and K. L. H. Williams, *J. Org. Chem.*, 1973, **38**, 1542.
[16] W. F. Gray, G. L. Deets, and T. Cohen, *J. Org. Chem.*, 1968, **33**, 4352.
[17] J. R. Hanson, J. Hawker, and A. F. White, *J.C.S. Perkin I*, 1972, 1892.

The labelling at C-4 and C-14 is in accord with the earlier ^{14}C results.[3] However, the enrichment at C-8 and not C-10 contrasts with the earlier result and leads to the conclusion that the farnesyl pyrophosphate is folded in the manner (3).

EXPERIMENTAL

General experimental details have been described previously.[17] The ^{13}C n.m.r. spectra were determined on a JEOL PFT-100 Fourier transform spectrometer operating at 25·15 MHz. The spectral width was 250 p.p.m., the pulse length 7 μs at a pulse interval of 1·5 s. The samples (100—250 mg) were dissolved in deuteriochloroform (0·5 ml), and the solvent deuterium provided the lock signal. Tetramethylsilane was used as an internal standard. The shifts are estimated to be accurate to 0·1 p.p.m. For the assignments 3000—5000 accumulations with 8192 data points were used whilst for the biosynthetic experiments 38,000 accumulations were used for both the unenriched control and enriched sample (75 mg each) which were determined consecutively.

[2-^{13}C]*Mevalonic Acid Lactone.*—[2-^{13}C]Acetic acid (1·5 g; 91·5% enrichment) was heated to reflux with bromine (1·5 ml) and red phosphorus (30 mg). The colourless solution was cooled and freshly prepared diazomethane in ether was added until the yellow colour persisted. The solvent was removed by careful distillation. The residue was distilled at room temperature by lowering the pressure to 10^{-3} mmHg and the distillate was dried and stored over Linde 4A molecular sieve to afford [2-^{13}C]methyl bromoacetate (3·1 g), τ 6·20 (3H, s, OMe), and 6·00 (2H, d, $J_{^{13}C-H}$ 144 Hz, 2-H). The methyl bromoacetate (3·1 g) and freshly redistilled 4-chlorobutan-2-one (2·4 g) in sodium-dried, analytical reagent grade ether (10 ml) was added to freshly activated 40 mesh zinc (2·2 g) under nitrogen. The suspension was stirred at 40° for 16 h. The solution was cooled and treated with saturated ammonium chloride solution (10 ml) for 1 h. The solution was extracted with ether, the extract was dried, and the solvent was evaporated off to give an oil which was distilled at 40° and 0·06 mmHg, to afford methyl [2-^{13}C]-5-chloro-3-hydroxy-3-methylpentanoate (1·5 g), $\nu_{max.}$ 3500, 2980, 1725, 1440, 1220, and 720 cm^{-1}, τ 8·80 (3H, s, 3′-H), 8·08 (2H, m, 4-H), 7·55 (2H, d, $J_{^{13}C-H}$ 128 Hz, 2-H), 6·52 (2H, m, 5-H), and 6·38 (3H, s, OMe). The chloroester was dissolved in dimethylformamide (3·2 ml) containing water (0·67 ml) and 5N-potassium hydroxide (3·2 ml) was added over 3 h at 50°. After a further 2 h, 20% hydrochloric acid was added to bring the pH to 2. The solution was stirred for 10 min and then poured into chloroform (200 ml) containing magnesium sulphate. After being stirred for 1 h, the solution was filtered and the solvents were evaporated off at room temperature *in vacuo* (1 mmHg) until the i.r. spectrum showed no absorption at 1664 cm^{-1} due to dimethylformamide. The residue was then distilled to afford [2-^{13}C]mevalonic acid lactone (750 mg) b.p. 100° at 0·1 mmHg, $\nu_{max.}$ 3400, 2980, 1720, 1410, 1270, 1240, and 940 cm^{-1}, τ 8·60 (3H, s, 3′-H), 8·08 (2H, m, 4-H), 7·40 (2H, d, $J_{^{13}C-H}$ 128 Hz, 2-H), 6·05 (1H, s, OH), and 5·4 (2H, m, 5-H), $\delta_{^{13}C}$ (p.p.m. from Me_4Si), 29·2 (q, 1%, 3′-C), 35·55 (t, 1%, 4-C), 44·5 (t, 90%, 2-C), 66·4 (t, 1%, 5-C), 671 (s, 1%, 3-C), 171·8 (s, 1%, 1-C). The mevalonic acid lactone was identified by comparison (i.r., t.l.c.) with authentic material.

Incubation with Trichothecium roseum.—*Trichothecium roseum* (IFO 6157) was grown on surface culture in Thomson bottles (750 ml broth) as described previously.[1,4] [2-^{13}C,2-^{14}C]Mevalonic acid (300 mg; 90% ^{13}C, 25 μCi ^{14}C) in ethanol (2 ml) was distributed between four bottles *Trichothecium roseum* 3 days after inoculation. After a further 5 days growth, the broth was extracted with chloroform and the trichothecin (100 mg), m.p. 117—118° (1·4 × 10^3 disint. min^{-1} mg^{-1}), was isolated by p.l.c. on silica in 30% ethyl acetate–light petroleum. The trichothecin in methanol (1 ml) was treated with 1N-potassium hydroxide in methanol (4·2 ml) at 0° and the solution was allowed to reach room temperature over 3 h. Water (5 ml) was added and the methanol was removed *in vacuo*. The solution was extracted with chloroform, and the extract was dried and evaporated to give a gum which was crystallized from ethyl acetate–light petroleum to afford trichothecolone (75 mg), m.p. 182—183° (1·76 × 10^3 disint. min^{-1} mg^{-1}) (lit.,[9] m.p. 183—184°) identified by comparison (i.r. and t.l.c.) with an authentic sample. The ^{13}C enrichment data are given in Table 2.

TABLE 2

^{13}C Enrichment of trichothecolone

	Unenriched sample		Enriched sample		
Carbon atom	Peak height (mm)	Peak height normalized onto C-12	Peak height (mm)	Peak height normalized onto C-12	Difference
2	98	2·39	86	2·26	−0·13
3	84	2·05	78	2·05	0
4	108	2·63	132	3·47	+0·84 (i)
5	38	0·95	34	0·89	−0·05
6	43	1·05	39	1·01	−0·04
7	85	2·05	73	1·92	−0·13
8	41	1·00	50	1·32	+0·32 (ii)
9	45	1·10	41	1·08	−0·02
10	113	2·76	101	2·66	−0·10
11	108	2·63	98	2·58	−0·05
12	41	1·00	38	1·00	0
13	86	2·10	82	2·15	+0·05
14	56	1·37	69	1·82	+0·45 (iii)
15	56	1·37	50	1·32	−0·05
16	62	1·51	57	1·50	−0·01
Noise level	5	0·12	5	0·13	0·01

% Incorporation (i) 0·35%; (ii) 0·35%; (iii) 0·36%.

We thank Dr. D. Shaw (Varian Associates) for some preliminary ^{13}C n.m.r. measurements and Mrs. A. Ward for growing the fermentations.

[3/2536 *Received*, 13*th December*, 1973]