Studies in Terpenoid Biosynthesis. Part 26.[1] Applications of ^{2}H and ^{13}C N.M.R. Spectroscopy to the Biosynthesis of the Illudin Sesquiterpenoids

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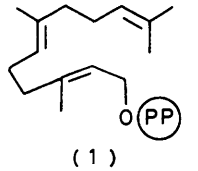
The ^{13}C n.m.r. resonances of the illudins have been assigned. The labelling pattern of illudin M and illudin S derived from [1,2-$^{13}C_2$]acetate and the induced coupling between C-6 and C-7 in samples biosynthesized from [1-^{13}C]acetate are in accord with the previous dissection of the carbon skeleton into isoprene units. The enrichment from [2-$^{2}H_3$]acetate and [2-$^{2}H_2$]mevalonate indicates that one mevalonoid label is lost from C-12 during the biosynthesis. The retention of a ^{2}H–^{13}C coupling at H-6 in illudin M which was biosynthesized from [5-$^{2}H_2$,5-^{13}C]mevalonate shows that this hydrogen atom is not involved in a hydride rearrangement in contrast to previous conclusions.

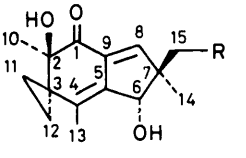
ILLUDIN M (2) and illudin S (3) [2] are sesquiterpenoid metabolites of the Basidiomycete, *Clitocybe illudens* (*Omphalotus olearus*).[3] Their carbon skeleton may be formed by cyclization of farnesyl pyrophosphate (1) *via* an intermediate related to humulene. In this context the carbon skeleton is related to those of a number of other sesquiterpenoids such as the marasmanes, sterpuranes, and lactaranes which are also found in metabolites of the Basidiomycetes.[4] Previous radioisotopic studies on the biosynthesis of illudins M and S have located [5] the centres which were labelled by [2-^{14}C]mevalonic acid and defined [6] the number of mevalonoid hydrogen atoms which were incorporated into illudin M. In this paper we present some results on the incorporation of ^{13}C and ^{2}H labelled substrates into illudin M, which, combined with n.m.r. methods to locate the sites of labelling, shed some further light on the biosynthesis.[7]

As a prelude to the application of ^{2}H and ^{13}C labelling studies to the biosynthesis, the ^{1}H and ^{13}C n.m.r. signals from the illudins were assigned. The ^{13}C n.m.r. resonances of illudins M (2) and S (3) were assigned (see Table 1) utilizing their multiplicity in the single-frequency off-resonance decoupled spectra and the variations of chemical shift in a number of derivatives. The most useful derivative was the 6-ketone (5). The olefinic signals (C-4 and C-5) then become part of an αβ-unsaturated ketone and show characteristic changes in chemical shift. The singlet resonance associated with C-7 shows a downfield shift compared to the parent alcohol. The cyclopropane signals (C-11 and C-12) were distinguished by the use of the upfield shift reagent, $Pr(fod)_3$ † with 6-acetoxyilludin M (4) (see Table 2). The cyclopropane resonance at δ 5.9 showed the greater upfield shift and was therefore assigned to the carbon atom *syn* to the 2-hydroxy-group (i.e. C-11). This signal which is the higher field of the two cyclopropane methylene resonances, is also experiencing the greater γ-shielding from the adjacent *syn*-hydroxy-group.[8] The carbon and proton resonances were correlated using the Birdsall–Feeney technique.[9] The cyclopropane signals at δ (^{1}H) 0.37 and 0.78 were associated with the carbon signal at δ (^{13}C) 8.6 p.p.m. and those at δ (^{1}H) 0.91 and δ 1.07 with the signal at δ (^{13}C) 5.9 p.p.m. The methyl group ^{1}H and ^{13}C resonances were also assigned by this method. Firstly the ^{1}H and ^{13}C n.m.r. signals were related to each other. Since the CH_3·C(OH) and CH_3·C=C proton resonances were readily assigned, the corresponding carbon signals could be identified. The carbon signals associated with C-14 and C-15 could then be distinguished on the basis of the *syn*-shielding of the C-6 γ-hydroxy-group.[8] Thus the methyl group resonance, δ (^{13}C) 14.1 p.p.m. (C-13), is associated with a proton signal, δ (^{1}H) 1.64, the δ (^{13}C) 20.6 p.p.m. (C-14) signal with δ (^{1}H) 1.16, the δ (^{13}C) 24.8 p.p.m. (C-10) with δ (^{1}H) 1.31, and δ (^{13}C) 27.2 p.p.m. (C-15) with δ (^{1}H) 1.06.

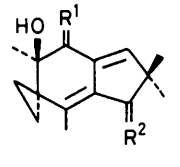


(1)

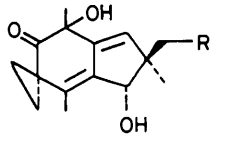
PP = pyrophosphate



(2) R = H
(3) R = OH



(4) R^1 = O; R^2 = α–OAc, β–H
(5) R^1 = R^2 = O
(6) R^1 = OH, H; R^2 = α–OH, β–H



(7) R = OH
(8) R = H

These assignments are consistent with the chemical shift variations typically associated with the structural changes (*cf.* illudin M and S, see Table 3).

Sodium [1,2-$^{13}C_2$]acetate, diluted with unlabelled material and containing [2-^{14}C]acetate tracer, was then

† $Pr(fod)_3$ = tris-(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyloctane-3,5-dionato)praseodymium.

TABLE 1

^{13}C N.m.r. resonances of some illudin derivatives (in $CDCl_3$, p.p.m. from Me_4Si)

Carbon atom	Compound (2)	(3)	(4) [a]	(5)	(6)	(7)
1	200.5	201.5	200.0	198.9	74.0 [b]	76.2
2	76.0	76.4	76.0	75.5	73.4 [b]	210.7
3	31.6	32.0	31.5	33.8	30.6	30.8
4	134.3	134.4	135.1	151.2	139.0	139.5 [b]
5	138.6	138.3	135.4	129.4	139.0	141.5 [b]
6	78.8	74.4	78.8	206.6	79.9	79.4
7	49.0	55.3	48.8	51.5	47.6	48.1
8	146.6	142.6	146.1	141.7	134.4	136.2
9	132.9	136.0	133.5	134.8	131.2	126.8
10	24.8	24.6	24.7	25.0	22.3	30.8
11	5.9	5.9	6.0	7.3	5.7	12.6
12	8.6	8.6	8.8	11.7	8.0	19.7
13	14.1	13.9	14.4	12.8	13.5	12.6
14	20.6	16.0	20.6	22.9	19.2	21.0
15	27.2	68.5	26.8	22.9	28.1	28.3

[a] Acetate signals, 20.6, 170.6. [b] These assignments may be interchanged.

TABLE 2

Variations of the ^{13}C signals of (4) in the presence of $Pr(fod)_3$

Carbon atom	Molar ratio, $Pr(fod)_3$: (4) 0 : 1	0.04 : 1	0.28 : 1	0.55 : 1
11	5.9	5.6	2.5	0.1
12	8.7	8.4	6.0	3.9
13	14.4	14.1	12.4	10.6
14	20.7	20.4	17.7	14.2
Ac	20.7	20.4	18.5	16.5
10	24.8	24.0	18.7	13.9
15	26.7	26.5	24.7	23.0
3	31.5	30.8	26.3	22.2
7	48.8	48.5	46.7	44.6
2	76.0	74.7	65.3	56.8
6	78.8	78.5	76.0	73.2
9	133.5	133.0	129.9	127.0
4	135.3	134.9	130.9	127.3
5	135.3	134.9	132.0	129.3
8	146.0	145.7	142.9	140.2
Ac	170.6	170.2	166.8	162.2
1	200.0	198.2	185.2	173.3

TABLE 3

^1H N.m.r. signals of illudins M (2) and S (3) and isoilludin S (7) (determined at 250 and 360 MHz in $CDCl_3$)

Proton	Compound (2)	(3)	(7) [a]
6	4.35	4.68	4.60
8	6.50	6.50	5.82
10	1.31	1.37	1.51
11	0.91	n.a. [b]	1.13
	1.07	n.a.	1.31 (2 H)
12	0.37	n.a.	1.81
	0.78	n.a.	
13	1.64	1.68	1.68
14	1.16	1.19	1.18
15	1.06	3.38	3.35 (*J* 11 Hz)
		3.48	3.47

[a] In $CDCl_3$–CD_3OD. [b] n.a. = not assigned.

fed to the fungus *Clitocybe illudens*, at the onset of metabolite production which occurs after 4 weeks growth on surface culture. The illudins M (2) and S (3) were isolated after a further 3 weeks growth. The ^{13}C–^{13}C n.m.r. coupling patterns are shown in the Figure. The higher field of the two cyclopropane methylene resonances (C-11) was coupled to C-3 thus defining the stereochemistry of folding of farnesyl pyrophosphate in this portion of the molecule. In particular if a protoilludane is a precursor of the illudins, the cyclopropane ring must be formed by migration of the C(4)–C(12) bond on the α-face of the molecule. The small ^{13}C–^{13}C coupling constant (13.7—15.3 Hz) found on the cyclopropane ring of the illudins has also been observed for other cyclopropane systems.[10]

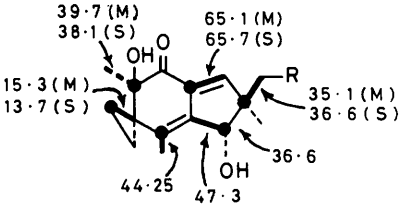


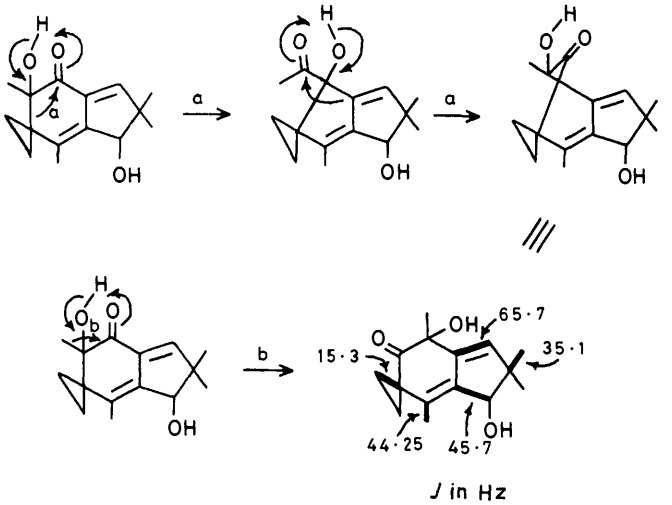
FIGURE The labelling patterns of illudin M (R = H) and illudin S (R = OH) biosynthesized from [1,2-$^{13}C_2$]acetate and [1-^{13}C]acetate: — denotes pairs of coupled atoms in the $^{13}C_2$ experiment (*J* in Hz); ● denotes enriched atoms in the 1-^{13}C experiment

Sodium [1-^{13}C]acetate was fed in one batch to *Clitocybe illudens* at the time of metabolite production in order to generate multiply labelled species. Apart from the expected enrichments of the metabolites, shown in the Figure, there was an induced coupling (*J* 36.6 Hz) between C-6 and C-7 arising from farnesyl pyrophosphate precursor molecules which were formed from more than one labelled unit. Since both C-1 and C-11 of farnesyl pyrophosphate are labelled by C-1 of acetate, this induced coupling would be compatible with a biosynthetic scheme in which C-6 originates from C-1 and C-7 from C-11 of farnesyl pyrophosphate.

The availability of a sample of illudin M labelled with carbon-13 permitted an investigation of the illudin–isoilludin rearrangement.[2] This rearrangement, which is catalysed by alumina, may either involve a double pinacolic shift (pathway a, Scheme 1) or a 1,2-methyl group migration (pathway b, Scheme 1). The former would retain the coupling between the ring A methyl group (C-10) and the tertiary carbinol (C-2) which is present in the illudin M labelled by [1,2-$^{13}C_2$]acetate. On the other hand this coupling would be destroyed in pathway b. In the event the resultant isoilludin M (7) had lost this coupling although the other couplings in the molecule remained intact. Hence, despite the reaction conditions, this rearrangement is a simple 1,2-shift.

The biosynthetic studies with [2-$^3H_2$,2-^{14}C]mevalonate revealed[6] the incorporation of only three mevalonoid atoms per molecule of illudin M instead of the expected four. Degradation had suggested the possible loss of a label from the C-12 position during the biosynthesis. An explanation for this can be presented in terms of a non-concerted cyclization (Scheme 2). An attempt has been made to re-investigate this aspect of illudin biosynthesis using [2-$^2H_3$]acetate and [2-$^2H_2$]mevalonate.

The signals in the ^2H n.m.r. spectrum of illudin M (see Table 4) at δ 0.38 (cyclopropane), 1.06, 1.16, 1.38, and 1.64 (C–Me), and 6.4 p.p.m. (=C–H) were enriched with use of [2-$^2H_3$]acetate whilst those at δ 0.38 and 1.16 p.p.m. were enriched with use of [2-$^2H_2$]mevalonate. In neither case was there a significant enrichment of the δ 0.78 p.p.m. cyclopropane signal, *i.e.* only one of the two protons (deuterons) attached to C-12 was apparently



Scheme 1 Mechanisms for the illudin–isoilludin rearrangement

enriched. Isoilludin S (7) is a crystalline derivative of illudin S which is more easily purified than illudin S; in this instance the illudin S would not crystallize and so it was converted into isoilludin S. The material derived from [2-$^2H_3$]acetate showed enrichment of the signals at δ (^2H) 1.21 (C–Me), 1.38 (cyclopropane), 1.55 and 1.70 (C–Me), 3.30 (CH_2OH), and 5.79 p.p.m. (=C–H). Although a quantitative interpretation of the integrals was not possible, nevertheless qualitatively it is apparent that the deuterium was not distributed between the signals in simple ratios. Although this may be a reflection of the low enrichments and hence high contribution from instrumental noise, it may also reflect isotope effects. For example the olefinic signals in both illudin M and isoilludin S showed a lower enrichment (*ca.* 50% of the expected value) from [2-$^2H_3$]acetate than the other signals. In these cases fully deuteriated substrates are involved and hence any isotope effect in breaking a C–D bond will be reflected in the incorporation of the other deuterium attached to that carbon.

In our previous work [6] we had found that C-6 (C-3′) was labelled by a (5*R*)-5-^3H-mevalonoid hydrogen atom. However when [1-^3H,2-^{14}C]farnesyl pyrophosphate was fed to the fungus, we had found that 90% of the tritium was lost. Unfortunately the carbon-14 incorporation was very low (0.02—0.03%) and consequently the carbon-14 label was not located by a subsequent degradation. Nevertheless we concluded from this result that the [(5*R*)-5-^3H]-mevalonoid hydrogen atom which was located at C-6 (C-3′) did not arise from the final isoprene

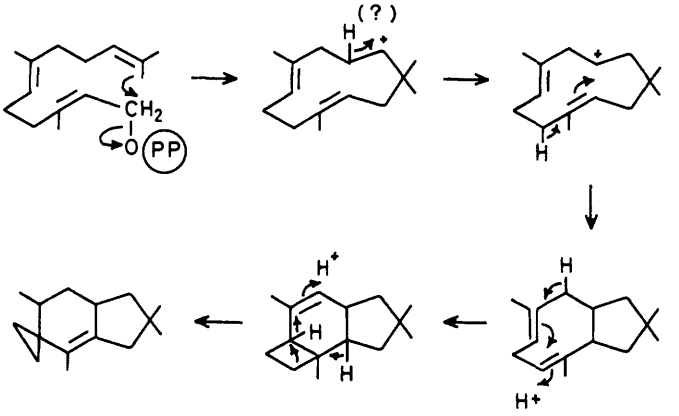
Table 4

Deuterium labelling patterns determined by ^2H n.m.r. spectroscopy

Substrate	^2H N.m.r. signal	Rel. int.	^1H N.m.r. signal	Assignment
[^2H]Acetate				
Illudin M (2)	0.38	0.9	0.37	12-H
	1.06		1.06	15-H
	1.16		1.16	14-H
	1.38	11.5	1.31	10-H
	1.64		1.64	13-H
	6.4	0.5	6.5	8-H
Isoilludin S (7)	1.21	4.25	1.18	14-H
	1.38	1	1.31	n.a.
	1.55	3.7	1.51	10-H
	1.70	4.6	1.68	13-H
	3.30	2	3.35, 3.47	15-H
	5.79	0.4	5.82	8-H
[2-^2H]Mevalonate				
Illudin M (2)	0.40	0.9	0.38	12-H
	1.20	2.0	1.16	14-H
[5-$^2H_2$,5-^{13}C]Mevalonate Illudin M (2)	0.9—1.0	*ca.* 2	0.78—0.91	11-H
	4.31 (*J* 23 Hz)	*ca.* 1	4.35	6-H

unit of farnesyl pyrophosphate and we proposed a series of hydride shifts to accommodate this unexpected result. Recently, in connection with fommanosin biosynthesis, this conclusion has been challenged.[11] Some doubt was also cast on our earlier findings by our observation that the ^{13}C label from [1,2-$^{13}C_2$]farnesyl pyrophosphate was scrambled in illudin M. A degradation–resynthesis pathway could account for a loss of tritium and a low incorporation of carbon-14. We have therefore re-investigated the labelling of C-6 using [5-$^2H_2$,5-^{13}C]-mevalonate. If the mevalonoid labels remain joined the ^{13}C–^2H coupling will be retained between C-6 and H-6 whilst if a hydride shift occurs, this coupling will be destroyed. Since deuterium has a spin of 1 these couplings are best observed in the ^2H n.m.r. spectrum.

[5-$^2H_2$,5-^{13}C]Mevalonic acid was prepared as follows.[12] Methyl [1-^{13}C]acetate was condensed with 1,1-dimethoxybutan-3-one and the resultant methyl [1-^{13}C]-



Scheme 2 Possible biosynthesis of illudins from farnesyl pyrophosphate

TABLE 5

Feeding experiments to *Clitocybe illudens*

Precursor	Quantity (μC; ^{14}C) [a]	Vol. of fermentation	Period of incubation: week—week	Mass of metabolite isolated	% Incorp. ^{14}C
[1,2-$^{13}C_2$]Acetate	180 mg (50 μCi)	2.25 l	4—7	450 mg of (2) 20 mg of (3)	4
[1-^{13}C]Acetate	1 g	2.25 l	4—7	90 mg of (2) 23 mg of (3)	—
[2-$^{2}H_3$]Acetate	4.38 g (150 μCi)	2.26 l	3—5	217 mg of (2) 388 mg of (3)	0.72 0.44
[2-$^{2}H_2$]Mevalonate	690 mg (20 μCi)	4.5 l	3—5	100 mg of (2)	0.35
[5-$^{2}H_2$,5-^{13}C]Mevalonate	504 mg (16 μCi)	4.5 l	3—5	192 mg of (2) 115 mg of (3)	0.34 0.06

[a] [2-^{14}C]-acetate or -mevalonate was added to the relevant precursors.

3-hydroxy-5,5-dimethoxy-3-methylpentanoate was then reduced with [$^{2}H_4$]lithium aluminium hydride. Hydrolysis of the dimethoxy acetal and oxidation then gave the [5-$^{2}H_2$,5-^{13}C]mevalonic acid which was fed to *Clitocybe illudens*. Although the enrichment of the resultant illudin **M** was very low, nevertheless the ^{2}H n.m.r. signal at δ 4.31 p.p.m. (H-6) appeared as a doublet, *J* 23 Hz, whilst the methylene signals at δ 0.9 p.p.m. were an unresolved multiplet. Consequently the 5-^{2}H mevalonoid label at C-6 has remained attached to the 5-^{13}C mevalonoid carbon atom and there is no need to invoke a at C-6 occurs with retention of configuration, there is a series of hydride shifts. Given that the hydroxylation net inversion of configuration at C-6 in the displacement of the pyrophosphate of farnesyl pyrophosphate and the formation of the C(6)–C(7) bond. These results can be accommodated in the biosynthetic Scheme 2.

EXPERIMENTAL

General experimental details have been described previously.[13] Carbon-13 n.m.r. spectra were determined at 25 MHz on a JEOL PFT-100 spectrometer and at 90.56 MHz on a Bruker WH 360 instrument. ^{2}H N.m.r. spectra were determined at 30.7 MHz (Varian XL-200), 38.4 (Bruker WH-250), and 55.28 MHz on a Bruker WH 360 spectrometer. High-field ^{1}H n.m.r. spectra were determined at 220 MHz (R 34, P.C.M.U., Harwell), 250MHz (Bruker WH 250), and at 360 MHz (Bruker WH 360).

General Fermentation Conditions.—*Clitocybe illudens* (ATCC 11719) was grown on surface culture in Thompson bottles on a yeast–peptone medium [6] (750 ml). The ages of the cultures at the time of feeding and harvest are given in Table 5. The precursors were administered in ethanol or water. The mycelium was filtered and the broth was saturated with sodium chloride, acidified to pH 2 with dil. hydrochloric acid and extracted with ethyl acetate. The extracts were separated into acidic and neutral fractions with aqueous sodium hydrogencarbonate. The illudin M and S were obtained from the neutral fractions by preparative layer chromatography on silica with methanol–chloroform (1 : 9) as eluant or column chromatography on silica with chloroform–ethyl acetate as eluant. The metabolites were identified by their m.p., and i.r. and n.m.r. spectra.

Conversion of Illudin M into Isoilludin M.—Illudin M (2) (45 mg) (from the [1,2-$^{13}C_2$]acetate experiment) in ethyl acetate (6 ml) was heated under reflux with alumina (0.2 g) (Woelm grade 1) for 6 h. The solution was cooled and the product recovered in ethyl acetate and purified by preparative layer chromatography to afford isoilludin M (20 mg) which was identified by its ^{1}H n.m.r. spectrum.[2] Isoilludin S (7), prepared similarly, had m.p. 184—186 °C, (lit.,[2] 179—180 °C) (Found: C, 67.9; H, 7.6. Calc. for $C_{15}H_{20}O_4$. C, 68.2; H, 7.6%).

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