Studies in Terpenoid Biosynthesis. Part XV.[1] Biosynthesis of the Sesquiterpenoid Illudin M

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The labelling pattern has been determined for the sesquiterpenoid illudin M, biosynthesized by *Clitocybe illudens* from [2-^{3}H,2-^{14}C]-, [(2*R*)-2-^{3}H,2-^{14}C]-, [(2*S*)-2-^{3}H,2-^{14}C]-, [(4*R*)-4-^{3}H,2-^{14}C]-, [5-^{3}H,2-^{14}C]-, and [(5*R*)-5-^{3}H,2-^{14}C]-mevalonic acids. Results from [5-^{3}H]mevalonate and [1-^{3}H]farnesyl pyrophosphate suggest that a hydride shift from C-9 to C-1 occurs; the results from [2-^{3}H]mevalonate suggest that a hydrogen atom is lost from C-4 of farnesyl pyrophosphate during the cyclization sequence.

SESQUITERPENOID substances may be classified[2] in terms of the mode of initial cyclization of farnesyl pyrophosphate (1). In the trichothecane series the cyclization has been shown to take place with involvement of the central and distal double bonds.[3] In contrast it has been suggested[4] that a humulene (2) may form the first cyclic precursor of the illudin series, as for example illudin M (5). The formation of this precursor requires cyclization of all-*trans*-farnesyl pyrophosphate by reaction at the distal double bond. The biogenetic proposals then involve protonation of the humulene (2) to initiate the further cyclization and rearrangement (3) which generates the illudin skeleton (4). The [2-^{14}C]mevalonate labelling pattern, which is illustrated in (5), is in accord with this biogenetic scheme.[5] Terpenoids retain mevalonoid hydrogen atoms until the late stages of their biosynthesis. Further-

[1] Part XIV, R. Evans and J. R. Hanson, *J.C.S. Perkin I*, 1976, 326.

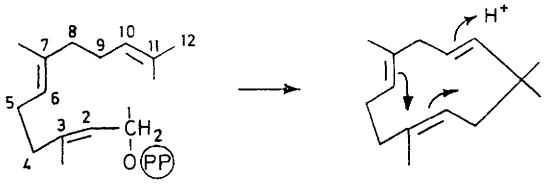
[2] W. Parker, J. S. Roberts, and W. Ramage, *Quart. Rev.*, 1967, **21**, 331.

[3] E. R. H. Jones and G. Lowe, *J. Chem. Soc.*, 1960, 3959; R. Achini, B. Muller, and Ch. Tamm, *Chem. Comm.*, 1971, 404; B. Achilladelis, P. M. Adams, and J. R. Hanson, *J.C.S. Perkin I*, 1972, 1425; J. R. Hanson and T. Marten, *ibid.*, 1974, 857; J. R. Hanson, T. Marten, and M. Siverns, *ibid.*, p. 1033.

[4] T. C. McMorris and M. Anchel, *J. Amer. Chem. Soc.*, 1965, **87**, 1594.

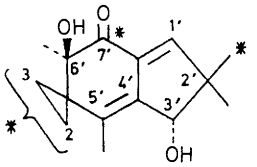
[5] M. Anchel, T. C. McMorris, and P. Singh, *Phytochemistry*, 1970, **9**, 2339.

more the chirality of mevalonoid hydrogen atoms in all-*trans*-farnesyl pyrophosphate has been established.[6] Consequently the location of the mevalonoid hydrogen atoms on the carbon skeleton of illudin M could provide



(1) (2)

(4) (3)



(5)

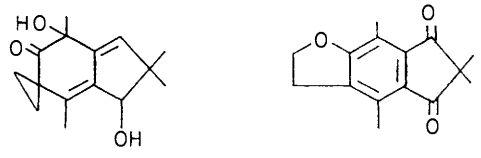
* label from [2-^{14}C]mevalonic acid

(PP) = pyrophosphate

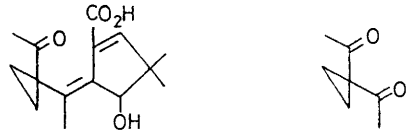
information on some of the intermediate stages in the biosynthesis. We now report [7] on the incorporation of mevalonoid hydrogen into illudin M by the fungus *Clitocybe illudens*.

Surface cultures of the fungus were grown for 6—8 weeks; antibacterial activity was then observed against *Staphylococcus aureus* and illudin M production occurred.[8] The precursors were added and the cultures were allowed to grow for a further 2—4 weeks. The illudin M was then isolated. Under our conditions of growth, illudin M was the major metabolite of this strain; other members of the illudin series were not isolated. The results from the variously labelled mevalonates are tabulated.

The illudin M (5) from the [(4*R*)-4-^{3}H,2-^{14}C]mevalonate experiment was converted into isoilludin M (6) (^{3}H : ^{14}C 3.0 : 1; atom ratio 0.90 : 3) with alumina.[8] Oxidation of the isoilludin M with chromium trioxide–pyridine afforded the dihydrobenzofuran (7)[9] with complete loss of tritium. Thus a [(4*R*)-4-^{3}H]mevalonoid label is located at C-1′. The illudin M (^{3}H : ^{14}C 5.95 : 1) from the [2-$^{3}H_2$,2-^{14}C]mevalonate experiment was oxidized with sodium periodate to the acid (8), which was then ozonised to afford the diacetylcyclopropane (9) which had lost two ^{14}C mevalonoid labels. The ^{3}H : ^{14}C ratio (6.1 : 1) corresponded to the presence of one rather than two [2-^{3}H]mevalonoid labels in this part of the molecule. In order to determine the stereochemistry of this loss of label, both [(2*R*)-2-^{3}H,2-^{14}C]mevalonate and [(2*S*)-2-^{3}H,2-^{14}C]mevalonate were fed to *Clitocybe illudens*. However neither showed a clear loss of label. Degradation of one sample from the [(2*S*)-2-^{3}H,2-^{14}C]mevalonate experiments to diacetylcyclopropane (9) with ruthenium dioxide and sodium periodate gave material with a ^{3}H : ^{14}C atom ratio of 0.77 : 1. The randomization and loss of label could occur through the action of prenyl isomerase or at a post-farnesyl pyrophosphate stage in the biosynthesis. In order to distinguish between these possibilities, all-*trans*-[4,8,12-$^{3}H_6$; 4,8,12-$^{14}C_3$]farnesyl pyrophosphate, prepared from [2-$^{3}H_2$,2-^{14}C]mevalonic acid using a pig-liver enzyme system,[10] was fed to *Clitocybe illudens*. The illudin M showed an ^{3}H : ^{14}C atom ratio of 3.4 : 3, *i.e.* the sum of the (2*R*)- and (2*S*)-mevalonate results. Consequently we are led to the



(6) (7)



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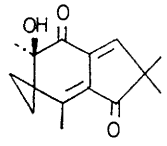


(10)

unexpected conclusion that there is a non-stereospecific loss of hydrogen at a stage after the formation of farnesyl pyrophosphate and that this is showing an isotope effect which would lead to some enrichment of tritium.

[6] G. Popjak and J. W. Cornforth, *Biochem. J.*, 1966, **101**, 553.

[7] Preliminary communication, J. R. Hanson and T. Marten, *J.C.S. Chem. Comm.*, 1973, 171.

[8] M. Anchel, A. Hervey, and W. J. Robbins, *Proc. Nat. Acad. Sci. U.S.A.*, 1950, **36**, 300; 1952, **38**, 927.

[9] K. Nakanishi, M. Ohashi, M. Tada, and Y. Yamada, *Tetrahedron*, 1965, **21**, 1231.

[10] C. Donninger and G. Popjak, *Proc. Roy. Soc.* 1966, *B*, **163**, 465; *Methods Enzymol.*, 1969, **15**, 446.

Illudin M incorporated three [5-^{3}H]mevalonoid labels. Oxidation with chromium trioxide in methylene chloride–pyridine afforded the diketone (10) with the loss of one label (^{3}H : ^{14}C ratio 4.12 : 1; atom ratio 1.95 : 3). A [5-^{3}H]mevalonoid label was therefore located at C-3′. Degradation with ruthenium dioxide–sodium periodate gave the diacetylcyclopropane (9) which retained two [5-^{3}H]mevalonoid labels (^{3}H : ^{14}C ratio 12.25 : 1; atom ratio 1.96 : 1). In order to determine the chiral origin of these labels, [(5*R*)-5-^{3}H,2-^{14}C]mevalonic acid, prepared from mevaldic acid by using mevaldate reductase,[10] was converted into all-*trans*-[1,5,9-^{3}H; 4,8,12-^{14}C]farnesyl pyrophosphate by using a pig liver enzyme system. This was then fed to *Clitocybe illudens*. The illudin M converted into [1-^{3}H,2-^{14}C]farnesyl pyrophosphate. However 90% of the tritium was lost when two different samples of [1-^{3}H,2-^{14}C]farnesyl pyrophosphate were incorporated into illudin M. Hence the [(5*R*)-5-^{3}H]-mevalonoid hydrogen atom which is located at C-3′ does not arise from the final isoprene unit of farnesyl pyrophosphate. Since the two [5-^{3}H]mevalonoid hydrogen atoms from the central isoprene unit have been located on the cyclopropane ring, the [(5*R*)-5-^{3}H]-mevalonoid hydrogen atom at C-3′ must originate in the starter unit.

These results may be rationalized as in the Scheme. Cyclization of C-1 onto the C-10 double bond of farnesyl pyrophosphate affords the cyclopropyl cation

Incorporation of substrates into illudin M by *Clitocybe illudens*

(a) [2-^{3}H]Mevalonate experiments

Substrate	[2-^{3}H$_2$, 2-^{14}C]-MVA		[(2*R*)-2-^{3}H, 2-^{14}C]-MVA	[(2*S*)-2-^{3}H, 2-^{14}C]-MVA		[4,8,12-^{3}H$_6$; 4,8,12-^{14}C$_3$]-Farnesyl pyrophosphate
^{3}H : ^{14}C ratio	9.6 : 1	11.1 : 1	9.8 : 1	9.05 : 1	8.34 : 1	12.44 : 1
Atom ratio	6 : 3	6 : 3	3 : 3	3 : 3	3 : 3	6 : 3
^{14}C activity fed	100 μCi	100 μCi	25 μCi	25 μCi	48 μCi	15 μCi
Illudin M ^{3}H : ^{14}C	4.7 : 1	5.9 : 1	6.0 : 1	5.3 : 1	4.81 : 1	6.9 : 1
Atom ratio	3.3 : 3	3.2 : 3	1.8 : 3	1.75 : 3	1.73 : 3	3.4 : 3
% Incorporation	0.06	0.03	0.7	0.6	0.6	0.05

(b) [4-^{3}H]Mevalonate experiment

Substrate	[(4*R*)-4-^{3}H,2-^{14}C]MVA
^{3}H : ^{14}C ratio	9.85 : 1
Atom ratio	3 : 3
^{14}C activity fed	100 μCi
Illudin M ^{3}H : ^{14}C	3.1 : 1
Atom ratio	0.95 : 3
% Incorporation	0.1

(c) [5-^{3}H]Mevalonate experiments

Substrate	[5-^{3}H$_2$, 2-^{14}C]-MVA	[1,5,9-^{3}H, 4,8,12-^{14}C]-Farnesyl pyrophosphate	[1-^{3}H, 2-^{14}C]-Farnesyl pyrophosphate	
^{3}H : ^{14}C ratio	12.65 : 1	10.3 : 1	14.8 : 1	12.8 : 1
Atom ratio	6 : 3	3 : 3	1 : 1	1 : 1
^{14}C activity fed	58.5 μCi	15 μCi	60 μCi	60 μCi
Illudin M ^{3}H : ^{14}C	6.10 : 1	6.1 : 1	1.45 : 1	1.5 : 1
Atom ratio	2.90 : 3	1.8 : 3	0.1 : 1	0.12 : 1
% Incorporation	1.2	0.1	0.02	0.03

retained two out of the three 5-*pro*-*R*-mevalonoid hydrogen atoms. Oxidation of another sample of illudin M, (^{3}H : ^{14}C ratio 7.3 : 1), prepared directly from [(5*R*)-5-^{3}H,2-^{14}C]mevalonic acid, gave the diketone (10) with a ^{3}H : ^{14}C ratio of 3.5 : 1 corresponding to the loss of one of the two tritium atoms. Hence the hydrogen atom at C-3′ arises from the 5-*pro*-*R*-position of mevalonate. Although this defines the chiral origin of this hydrogen atom, it does not define the prenyl group from which it originates. This was investigated as follows. [1-^{3}H,2-^{14}C]Farnesyl pyrophosphate[11] would provide a label corresponding to the [5-^{3}H]mevalonoid label of the final isoprene unit. [2-^{14}C]Methyl farnesoate was prepared from geranylacetone and [2-^{14}C]methyl bromoacetate and then reduced to [2-^{14}C]farnesol with lithium aluminium hydride. The farnesol was oxidized to farnesal with manganese dioxide and this was then reduced with sodium [^{3}H]borohydride before being (11). Hydride shifts serve to transfer the positive charge to C-9 (12), which then initiates further cyclization, possibly *via* the olefin (13). Molecular models show that as the 1-*pro*-*S*-hydrogen atom of farnesyl pyrophosphate migrates on one face of the cyclopropyl system, the 5-*pro*-*R*-mevalonoid hydrogen atom can migrate from C-9 on the opposite face leading to a net inversion of configuration at C-1 (= C-3′ of the illudin system). Subsequent hydroxylation then proceeds in the normal manner with retention of configuration to abstract the 1-*pro*-*R*-hydrogen atom of farnesyl pyrophosphate. As in other sesquiterpenoid biosyntheses such as those of longifolene[12] and culmorin,[13] which involve an eleven-membered ring intermediate, it is the 1-*pro*-*S*-hydrogen atom of farnesyl pyrophosphate which has migrated to C-10.

[11] G. Popjak, J. W. Cornforth, R. H. Cornforth, R. Rhyage, and D. S. Goodman, *J. Biol. Chem.*, 1962, **237**, 56.

[12] F. Dorn, P. Bernasconi, and D. Arigoni, *Chimia* (*Switz.*), 1975, **29**, 25.

[13] J. R. Hanson and R. Nyfeler, *J.C.S. Chem. Comm.*, 1975, 824.

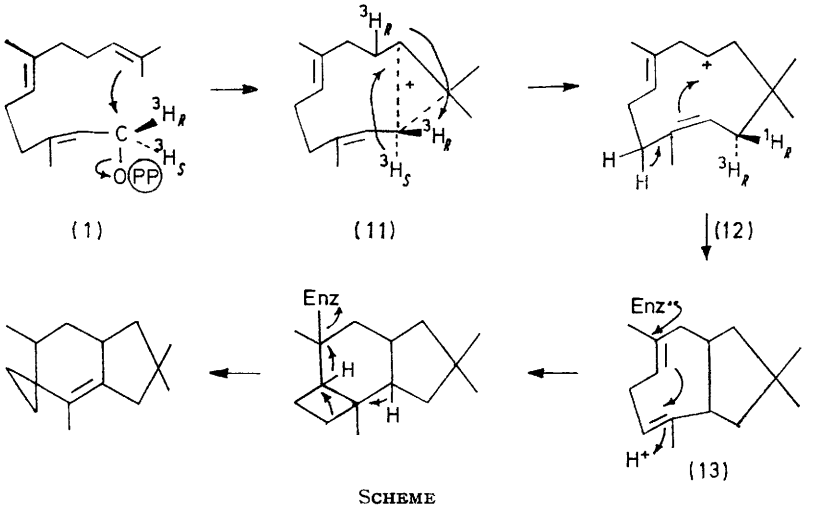
In illudin M dehydrogenation and hydroxylation steps have removed key hydrogen atoms on the five-membered ring. Consequently we are examining this type of cyclization in less highly oxidized fungal metabolites.

EXPERIMENTAL

General experimental details have been described previously.[14] *Clitocybe illudens* (ATCC 11719) was grown in surface culture in Thompson bottles (750 ml) on a medium containing (per litre) glucose (50 g), peptone (20 g), yeast extract (2 g), potassium dihydrogen phosphate (0.87 g), magnesium sulphate (0.5 g), calcium chloride (0.3 g), corn steep liquor (5 ml), and a trace metal solution (20 ml). The latter contained (per litre) manganese chloride (0.36 g), zinc chloride (0.2 g), iron(III) chloride (0.5 g), and copper sulphate (0.05 g). The fermentations were grown at 24 °C and harvested 8—10 weeks after inoculation. The culture filtrate was acidified to pH 2 and the metabolites were recovered in ethyl acetate. Chromatography of the neutral fraction on silica plates, developed in ethyl acetate–light petroleum (1 : 1), gave illudin M (*ca.* 90 mg l^{-1}) which crystallized from ethyl acetate as prisms, m.p. 129°, $[\alpha]_D^{20}$ −130° (*c* 0.3 in $CHCl_3$) (lit.,[4] 128—130°, $[\alpha]_D^{20}$ −126°) (Found: C, 72.5; H, 8.4. Calc. for $C_{15}H_{20}O_3$: C, 72.55; H, 8.1%). In the feeding experiments the labelled substrate (10—100 μCi ^{14}C) was dissolved in ethanol and distributed between 2—4 bottles of 6—8-week-old cultures of *Clitocybe illudens*, which were then harvested after a further 2—3 weeks. The results for the individual experiments are given in the Table.

Isoilludin M (4).—A solution of illudin M (240 mg) {from the [(4*R*)-4-^{3}H]mevalonate experiment} in ethyl acetate (22 ml) was heated under reflux in the presence of alumina (Woelm grade 1, neutral) (2 g) for 6 h. The solution was filtered and evaporated to give a gum which was chromatographed on silica. Elution with 60% ethyl acetate–light petroleum gave isoilludin M (160 mg), which crystallized from ethyl acetate as needles, m.p. 136—137°, $[\alpha]_D^{20}$ +84° (*c* 0.3 in $CHCl_3$) (lit.,[5] 136—137°), ^3H : ^{14}C 3.0 : 1.

Oxidation of Isoilludin M.—A solution of isoilludin M (125 mg; diluted with unlabelled material) in dry pyridine (2.5 ml) was treated with freshly prepared chromium trioxide–pyridine complex (375 mg) at 0 °C. The solution was slowly warmed to 50 °C, stirred at this temperature for 24 h, filtered, diluted with water, and extracted with ethyl acetate. The extract was washed with dilute hydrochloric acid and water, dried, and evaporated to give a gum which was chromatographed on silica. Elution with 50% ethyl acetate–light petroleum gave 2,3-*dihydro*-4,6,6,8-*tetramethylindeno*[5,6-b]*furan*-5,7-*dione* (7) (60 mg), which crystallized from ethanol as needles, m.p. 144—145° (Found: C, 73.6; H, 6.6. $C_{15}H_{16}O_3$ requires C, 73.75; H, 6.6%), $\nu_{max.}$ 2 860, 1 725, 1 688, and 1 570 cm^{-1}, $\lambda_{max.}$($CHCl_3$) 242, 259, and 287 nm (ε 6 710, 4 610, and 6 010), τ 8.77 (6 H, s), 7.47 (3 H, s), 7.40 (3 H, s), 6.76 (2 H, t, *J* 9 Hz), and 5.20 (2 H, t, *J* 9 Hz), 1.83 × 10^3 disint. min^{-1} mg^{-1} ^{14}C (no ^3H).



SCHEME

Oxidation of Illudin M to the Acid (8).—Sodium periodate (400 mg) in methanol (1.5 ml) and water (2.5 ml) was added to a solution of illudin M (200 mg) (from the [2-$^3H_2$]-mevalonate experiment) in methanol (1.5 ml) and the mixture was left at room temperature overnight. The product was recovered in ethyl acetate and purified by preparative t.l.c. on silica in ethyl acetate–light petroleum–acetic acid (50 : 50 : 1) to afford the acid (8) (100 mg), m.p. 213° (decomp.) (lit.,[4] 214—215°), ^3H : ^{14}C 5.9 : 1.

Ozonolysis of the Acid (8).—Ozonized oxygen was bubbled through a solution of the acid (8) (30 mg) in dichloromethane–pyridine (1 : 1; 4 ml) at −78 °C for 30 min. Nitrogen was blown through the solution while it attained room temperature. The solution was acidified with dilute hydrochloric acid and the diacetylcyclopropane (5 mg) (^3H : ^{14}C 6.1 : 1) (9) was recovered in ether and purified by preparative layer chromatography. It had i.r. and n.m.r. spectra identical with those of an authentic sample prepared from acetylacetone and ethylene.[15]

Oxidation of Illudin M with Ruthenium Dioxide.—Ruthenium dioxide (6 mg) was added to a solution of sodium periodate (1 g) in water (10 ml). Illudin M (16 mg) {from the [(2*S*)-2-^{3}H]mevalonate experiment} in carbon tetrachloride (15 ml) was then added and the mixture was stirred vigorously for 24 h. Propan-2-ol was then added, the mixture was filtered, and the phases were separated.

[14] J. R. Hanson, J. Hawker, and A. F. White, *J.C.S. Perkin I*, 1972, 1892.

[15] K. Ichikawa, O. Itoh, T. Kawamura, M. Fujiwara, and T. Ueno, *J. Org. Chem.*, 1966, **31**, 447.

The aqueous phase was extracted with carbon tetrachloride and the extracts were combined, dried, and evaporated. The residue was diluted with unlabelled 1,1-diacetylcyclopropane (10 mg) and purified by preparative layer chromatography. The 1,1-diacetylcyclopropane had $^{3}H : ^{14}C$ 6.40 : 1 (atom ratio 0.77 : 1). The illudin M from the [5-$^{3}H_2$]mevalonate experiment was oxidized as above.

Oxidation of Illudin M to the Diketone (10).—Dry chromium trioxide (60 mg) was added to a solution of dry pyridine (0.1 ml) in dry dichloromethane (5 ml). After 15 min, illudin M (25 mg) (from the [5-$^{3}H_2$]mevalonate experiment) was added. After a further 20 min, the solution was extracted with dilute hydrochloric acid, aqueous sodium hydrogen carbonate, and water, and evaporated *in vacuo*, and the diketone (10) was purified by preparative layer chromatography in ethyl acetate–light petroleum (1 : 1) on silica to give an oil, $^{3}H : ^{14}C$ 4.2 : 1. In one experiment *3'-oxoilludin M* (10) was obtained crystalline. It crystallized from pentane at 20 °C as needles, m.p. 65°, $[\alpha]_D^{20}$ −162° (*c* 0.1) (Found: C, 72.9; H, 7.4. $C_{15}H_{18}O_3$ requires C, 73.2; H, 7.3%), $\nu_{max.}$ 3 490, 1 710, 1 630, and 1 600 cm^{-1}, τ 9.5 (m, cyclopropyl H), 8.78, 8.75, 8.64, and 7.91 (each 3 H, s, CMe), 6.46 (1 H, s, OH), and 3.14 (1 H, s, 1'-H).

The illudin M from the [(5*R*)-5-^{3}H]mevalonate was oxidized as above.

We thank Mrs. A. Ward for growing the fermentations.

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