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THE BIOGENESIS OF ILLUDINS S AND M IN *CLITOCYBE ILLUDENS**

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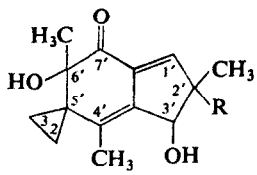
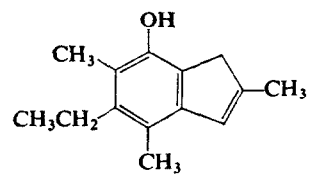
Abstract—Illudin S and illudin M were biologically labelled with C^{14} by adding mevalonic acid 2-C^{14} as precursor to cultures of *Clitocybe illudens*. The positions of the label, determined by degradation, are consistent with the origin of these sesquiterpenes from 3 molecules of mevalonic acid.

ILLUDIN S (I) and illudin M (II) incorporate mevalonic acid-2-C^{14} (cf. Ref. 1) when the latter was added to still cultures of *Clitocybe illudens*. We have now located the positions of the isotope by degradation of the labelled metabolites.

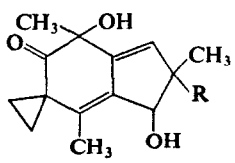
According to the proposed scheme of biogenesis of the illudins,[2] incorporation of mevalonic acid should result in labelling at one of the gem-dimethyl groups (or in the hydroxymethyl group) attached to carbon 2′. The other labelled carbons should be at position 2 or 3, and at position 7′.



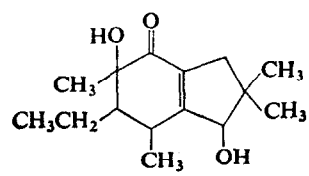
(I) R = CH_2OH
(II) R = CH_3



(III)



(IV) R = CH_2OH
(V) R = CH_3



(VI)

Labelled illudins were separated by countercurrent distribution of an extract of the culture liquid and recrystallized to constant count. Dilution of the isotope was 40,000–80,000 times. Degradation was carried out as described below. The results are summarized in Tables 1 and 2.

* Part V of a series: "Metabolic Products of *Clitocybe illudens*", for Part IV see M. S. R. NAIR, H. TAKESHITA, T. C. MCMORRIS and M. ANCHEL, *J. Org. Chem.* **34**, 240 (1969).

[1] E. LEETE, H. GREGORY and E. G. GROSS, *J. Am. Chem. Soc.* **87**, 3475 (1965).
[2] T. C. MCMORRIS and M. ANCHEL, *J. Am. Chem. Soc.* **87**, 1594 (1965).

TABLE 1. DISTRIBUTION OF LABELLED CARBON FROM MEVALONIC ACID -2-C^{14} IN ILLUDIN S

	cpm/mM × 10^{-3}		
(1) Parent compound	(2) Acetate (propionate) from Kuhn–Roth oxidation	2/1: Found	2/1: Calc.
Illudin S (I) 212	23·6	0·115	0·111 (1/9)
Isoill. S (IV) 221	52·0	0·234	0·222 (2/9)
Indenol (III) 210	30·9	0·147	0·167 (2/12)
	(69·0)	(0·328)	(0·333) (1/3)

TABLE 2. DISTRIBUTION OF LABELLED CARBON FROM MEVALONIC ACID 2-C^{14} IN ILLUDIN M

	cpm/mM × 10^{-3}		
(1) Parent compound	(2) Acetate (propionate) from Kuhn–Roth oxid.	2/1: Found	2/1: Calc.
Illudin M (II) 515	22·5	0·044	0·056 (1/18)
Isoill. M (V) 495	80·5	0·163	0·167 (1/6)
Tetrahydroill. M (VI) 490	13·1	0·027	0·125 (1/8)
	(150)	(0·31)	(0·33) (1/3)

Kuhn–Roth oxidation of illudin S afforded acetate derived from the methyl groups attached to carbons 2′, 4′, and 6′. Of these methyl groups the label would be expected only in that attached to carbon 2′. The acetate obtained, counted as its naphthylamine derivative,[2] contained per mole, 1/9 of the activity of the intact molecule, the result expected if only one of the acetate molecules was labelled and contained 1/3 of the total activity of the illudin molecule.

A small proportion of the label was in the CH_2OH group at carbon 2′, as determined by isolation of this moiety as formaldehyde, obtained in the conversion of illudin S to the indenol (III).[1] The formaldehyde, counted as its dimedone derivative, had 1·3%/mole (*ca.* 1/75) of the count of the illudin S from which it was derived. The indenol had the same activity as illudin S within the experimental error.

Kuhn–Roth oxidation of III afforded acetate from the methyl groups at 2′, 4′, and 6′ and from that of the ethyl side-chain. The methylene group of this side-chain would be obtained as the acetate carboxyl, or as carbon 2 of propionate. Acetic and propionic acids, separated by chromatography of the mixture in the form of their naphthylamine derivatives, were both active. The propionic acid/mole accounted for 1/3 of the activity of the original indenol, the expected value. The activity for the acetate/mole was near 2/12 that of the indenol, the expected value for two of four labelled acetate molecules, 1 label/molecule from each triply labelled molecule of indenol.

To obtain carbon 7′ for counting, illudin S was converted to isoilludin S (IV).[3] Kuhn–Roth oxidation of IV yielded acetate derived from methyl groups at 2′, 4′, and 7′. Activity of acetate/mole, now accounted for 2/9 of the activity of the intact molecule. The extra activity

[3] M. TADA, Y. YAMADA, N. S. BHACCA, K. NAKANISHI and M. OHASHI, *Chem. Pharm. Bull., Japan* **12**, 853 (1964).

of the acetate from IV, compared to that from I, can be due only to an active carbon at 7', now obtained in the carboxyl group of the acetate.

The degradation of illudin M was similar but not identical to that of illudin S. Kuhn–Roth oxidation of II should give 3 moles of acetate: 1 mole from the gem dimethyl, and 2 moles from the methyls at carbons 4' and 6'. If the label at 2' is, by analogy with illudin S, exclusively in one methyl of the gem-dimethyl group, if this is the only one labelled, and if both contribute equally to the acetate in the Kuhn–Roth, the expected activity of the acetate would be 1/18 (1/3 × 1/3 × 1/2) that of the molar activity of the parent illudin M. The value obtained was slightly low. This could be due to experimental error (*ca.* 20%) but may be attributable, at least in part, to the incomplete Kuhn–Roth oxidation at the gem-dimethyl. (The C-methyl value obtained in the Kuhn–Roth was about 80% of the theoretical.)

Carbon 7' was obtained for counting, similarly as for illudin S, as the carboxyl carbon of acetate from Kuhn–Roth of isoilludin M (V). The activity of the acetate was 1/6 that of the parent compound, the value expected if carbon 7' was labelled, and if the label in the gem-dimethyl group was exclusively in one of the methyl groups (1/3 × 1·5/3).

Illudin M did not give a single product using conditions under which illudin S gave the indenol III.[1] But hydrogenation in ethyl acetate gave a crystalline compound, $C_{15}H_{24}O_3$ (VI), in which the cyclopropane ring was opened to give an ethyl side-chain. (The structure VI was confirmed by analysis, spectral evidence, and by oxidation with MnO_2 to give the conjugated diketone ν_{max} 1684, 1705 cm^{-1}). Kuhn–Roth oxidation of VI gave acetate derived from four methyl groups, as well as propionate from the ethyl side-chain. The activity of the acetate, assuming no propionate formation, should be 1/8 (1/3 × 1·5/4) that of the parent tetrahydroilludin M. The found value was only about 44% of this. The result is not surprising since a large proportion of the ethyl side-chain was obtained as propionate in the Kuhn–Roth because of the mild conditions used. This, combined with possible incomplete oxidation at the gem-dimethyl carbon, would result in a low proportion of labelled acetate. The Kuhn–Roth value was, in fact, only about 55% of the theoretical when calculated on the basis of acetate. Clear demonstration that 33% of the activity of VI was in the ethyl side-chain, could nevertheless be obtained from the propionate derived from the Kuhn–Roth oxidation. The activity of this fragment, which is not affected by dilution with unlabelled propionate (since none is available from VI), was in good agreement with the expected 33% the activity of VI.

EXPERIMENTAL*

Isolation of Radioactive Illudins

Eight Fernbach flasks containing 1 l. each of cornsteep medium† and 4 g of glass wool, were inoculated with *Clitocybe illudens* and incubated at 25° for 6 weeks, at which time the mycelial mat covered the surface of the liquid. The flasks were reflooded with cornsteep medium and incubated for 8 days longer. To each flask was then added 10 μc of mevalonic acid 2-C^{14} (DBED salt, spec. act. 3·16 mc/mM), in 10 ml of sterile distilled water. After 5 weeks the culture liquid was harvested and extracted with ethyl acetate. The extract was taken to dryness *in vacuo*, and the residue was subjected to a 60 tube countercurrent distribution between $CHCl_3$ and H_2O. Tubes 36–51 yielded crystalline illudin S. A total of 1·8 g of crude material was obtained, which after two recrystallizations yielded 0·48 g of pure illudin S with a count of 800 counts/min/mg (212,000 counts/min/mM).

Illudin M was obtained from tubes 0–4. It was further purified by countercurrent distribution between

* All samples were counted by liquid scintillation (Nuclear Chicago Model 6810).

† Liquid cornsteep medium contains, per liter, 40 g dextrose, 10 ml cornsteep liquor (5 g solids, in syrup obtained from Corn Products Co., 10 E. 56th St., N.Y., U.S.A.) adjusted to pH 6, and 20 ml Dox mineral solution (contains per liter, 150 g $NaNO_3$, 50 g KH_2PO_4, 25 g KCl, 25 g $MgSO_4 \cdot 7H_2O$).

2:1 ether–Skelly B* as the upper phase and 1:1 MeOH–H_2O as the lower phase. Fractions 19–37 yielded 2·86 g of crystalline illudin M with a count of 2080 counts/min/mg (515,000 counts/min/mM). After two more crystallizations, 0·42 g of crystals, m.p. 132–133°, were obtained. The count was unchanged.

Kuhn–Roth Oxidation of Radioactive Illudin S(I)

20 mg illudin S (212,000 cpm/mM) was refluxed with 5 ml of a solution of 5 N chromic acid (4·175 g of chromic anhydride in 6·25 ml H_2SO_4, diluted to 25 ml with H_2O) for 90 min. Excess chromic acid was reduced by dropwise addition of 85% hydrazine hydrate to the ice-cold solution. Aq. NaOH (3 ml, 10%) was added, followed by phosphoric acid (0·3 ml). The resulting green solution was steam distilled until 400 ml of distillate was collected. Titration of this solution with NaOH showed 14·30 mg of acetic acid, equivalent to 17·31% methyl (in theory 17·04%). The resulting sodium acetate solution was taken to dryness *in vacuo* and treated with α-naphthylamine hydrochloride and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride according to the method of Leete *et al.*[1] The naphthylamine derivative was sublimed under reduced pressure and crystallized twice from benzene–ligroin to give colorless needles, m.p. 160–161°, with an activity of 23,600 counts/min/mM, unchanged by further recrystallization.

Radioactive Isoilludin S (IV)

A stirred solution of radioactive illudin S (152 mg, 212,000 counts/min/mM) in ethyl acetate (4 ml) was refluxed under N_2 with alumina (Brockmann, activity 1, 0·25 g) for 90 min. The alumina was filtered off and washed with EtOH. The combined filtrate and washings were taken to dryness *in vacuo* yielding 115 mg of a light yellow solid. After crystallization from ethyl acetate–ligroin, colorless needles, m.p. 180–181°, were obtained with an activity of 221,000 counts/min/mM, unchanged by further recrystallization.

Kuhn–Roth Oxidation of Radioactive Isoilludin S

This was carried out in the same way as described for illudin S, using 33 mg of radioactive isoilludin S (221,000 counts/min/mM). The distillate contained 22·25 mg of acetic acid, equivalent to 16·75% methyl (in theory 17·04%). The naphthylamine derivative, after sublimation and crystallization, melted at 160–161° and had an activity of 52,000 counts/min/mM, unchanged by further recrystallization.

Conversion of Radioactive Illudin S to the Indenol (III)

Illudin S (212,000 counts/min/mM, 200 mg) was refluxed in 10 ml of EtOH containing 1·6 g of granular Zn, and 0·1 ml of HI was added every 30 min for 5 hr. After removal of the Zn, the mixture was steam distilled until 400 ml of the distillate was collected. The crystals obtained from the distillate had m.p. 130–131°, and an activity of 210,000 counts/min/mM, both unchanged by recrystallization from ethyl acetate–ligroin.

The filtrate from the indenol was treated with a solution of 400 mg of dimedone in 5 ml of EtOH, and left in the refrigerator overnight. The formaldehyde dimedone derivative was filtered off and recrystallized from dilute alcohol; needles, m.p. 189–90°, which gave a single spot (R_f 0·31; compared to R_f 0·53 for the indenol in same solvent) on TLC (benzene–ethyl acetate, 99·5:0·5). It had an activity of *ca.* 9·5 counts/min/mg (2740 counts/min/mM).

Kuhn–Roth Oxidation of Radioactive Indenol (III)

The indenol (40 mg) was stirred with 5 ml of 5 N chromic acid for 22 hr at room temp. The solution was worked up in the same way as that from oxidation of illudin S. The acid obtained in the distillate was equivalent to 34·62 mg of acetic acid, or 21·1% methyl (in theory 29·7%) based on acetic acid.

The naphthylamine derivative, prepared as described,[1] was separated on a preparative silica gel TLC plate (R_f 0·26 and 0·47 for acetamido- and propionamido-derivatives respectively, ethyl acetate–ligroin 1:1). The bands were eluted separately and the acetamido- and propionamido-derivatives so obtained were sublimed *in vacuo*.

The acetamido-naphthalene melted 159–160° and had an activity of 30,900 counts/min/mM. After recrystallization from benzene-ligroin it melted at 160–161°. The count was not significantly changed. The propionamidonaphthalene melted at 114° and had an activity of 69,000 counts/min/mM. After recrystallization it melted at 119–120°. The count was not significantly changed.

Kuhn–Roth Oxidation of Radioactive Illudin M (II)

The reaction was carried out as described for illudin S. Titration of the steam distillate with NaOH showed 12·34 mg of acetic acid, equivalent to 14·55% methyl. (In theory, 18·2%.) The naphthylamine derivative, after sublimation and crystallization, melted at 158–159° and had an activity of 22,500 counts/min/mM, unchanged by further recrystallization.

* Essentially normal hexane fraction, b.p. 60–70°, obtained from Skelly Oil Co., 605 W. 47th St., Kansas City, Mo., U.S.A.

Radioactive Isoilludin M (V)

A solution of radioactive illudin M (52 mg, 515,000 cpm/mM) in 4 ml of ethyl acetate was refluxed under N_2 with alumina (Brockmann activity 1, 0·2 g) for 6 hr. The reaction mixture after removal of alumina was chromatographed on silica gel using ligroin with increasing amounts of ethyl acetate as eluting solvent. Isoilludin M (24 mg, m.p. 136–38°) was obtained in the 1:1 ligroin–ethyl acetate fraction. λ_{max}^{EtOH} 249 (ϵ 15,200); ν_{max} (KBr) 3448 (S), 2890 (W), 1653 (S), 1466 (S), 1445 (S), 1415 (S), 1313 (W), 1182 (W), 1104 (W), 952·4 (S), 918 (W), 806·5 (S), 730 (S), 682·7 (S) cm^{-1}; τ 4·18 (singlet, olefinic H), 5·7 (singlet, —C*H*OH), 6·37 (broad peak, 2-OH; disappears in D_2O), 8·34, 8·52, 8·83, 8·99 (all singlets 4-CH_3), 8·4–9·5 (multiplet, 4 cycloprane H). (Found: C, 71·77; H, 8·11; Calc. for $C_{15}H_{20}O$: C, 72·55; H, 8·12%.) It had an activity of 495,000 counts/min/mM, unchanged by recrystallization from ligroin.

Kuhn–Roth Oxidation of Radioactive Isoilludin M

The oxidation was carried out as described for illudin S. A 36·9 mg sample of isoilludin M yielded 20·79 mg of acetic acid in the distillate, equivalent to 14·25% methyl (in theory 18·2%). The naphthylamine derivative melted at 158–159° and had an activity of 80,500 counts/min/mM, unchanged by crystallization from benzene–ligroin.

Radioactive Tetrahydro-illudin M (VI)

A solution of illudin M (100 mg) in ethyl acetate (20 ml) containing 100 mg of Pd–C catalyst (5%) was hydrogenated until absorption of H_2 ceased (2 hr). The residue of the solution was subjected to preparative TLC on silica gel using ethyl acetate–ligroin 3:1. The major product, the slowest moving of four spots (R_f 0·48) was eluted and crystallized from ethyl acetate–ligroin, to give 20 mg of colorless needles, m.p. 183–184°, λ_{max}^{EtOH} 240 mμ ν_{max} 1680 and 1640 cm^{-1}, τ 3·28, 4·49 (broad, 2-OH disappear in D_2O), 5·37 (singlet, —C*H*OH), 6·9–8·4 (multiplet for 5 protons 2 CH_2 and one—CH—CH_2), 8·63, 8·75, 8·87, 8·98, 9·1 (5-CH_3); m.s. (75 eV), *m/e* (relative intensity) 253 ($M + 1^+$, 9) 252 (M^+, 48), 166 (67), 167 (95), 151 (100), 123 (34), 71 (33), 55 (29), 43 (87), 41 (36). (Found: C, 71·32; H, 9·55; O, 19·37. Calc. for $C_{15}H_{24}O_3$: C, 71·39; H, 9·59; O, 19·02%.) The activity of the product was 490,000 counts/min/mM unchanged by recrystallization.

MnO_2 Oxidation of VI

A $CHCl_3$ solution (20 ml) of VI (20 mg) was stirred for 3 hr at room temp. with active MnO_2 (50 mg). The MnO_2 was filtered off and the solvent evaporated under reduced pressure. The light-yellow residue showed λ_{max}^{EtOH} 257 mμ, ν_{max} (KBr) 1664, 1705 (W) cm^{-1}. After recrystallization from ethyl acetate–ligroin it melted at 144–145°.

Kuhn–Roth Oxidation of Radioactive Tetrahydro-illudin M

Tetrahydro-illudin M (68 mg, 490,000 counts/min/mM) was oxidized as described for the indenol (III). The acid obtained in the distillate was equivalent to 36·46 mg of acetic acid, or 13·31% methyl (in theory 23·8% based on acetic acid). The naphthylamine derivative, prepared as described[2] was separated into the acetamido and propionamido-derivatives, as described for the Kuhn–Roth oxidation product of the indenol. The acetamido-derivative melted at 158–159° and had an activity of 13,100 counts/min/mM, unchanged on recrystallization. The propionamido-derivative melted at 118–119° and had an activity of 150,000 counts/min/mM.

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