Biosynthesis of illudosin, a fomannosane-type sesquiterpene, by the Basidiomycete *Omphalotus nidiformis*

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Sites of incorporation of ^{13}C-labelled acetates and [1-^{13}C,2-$^{2}H_3$]acetate into illudosin **1**, a fomannosane type sesquiterpene produced by *Omphalotus nidiformis*, have been determined by ^{13}C NMR spectroscopy. The biosynthesis of illudosin was as expected, apart from the presence of some labelled species with two deuteriums attached to C-6 in illudosin which is also labelled at C-5 with ^{13}C. This unusual rearrangement was also observed in the co-metabolite illudin M. A mechanism is proposed which involves the formation of a series of carbocations at the active site of the cyclase enzyme that can be reprotonated (or deuterated) non-stereospecifically.

Introduction

The illudins belong to the largest group of sesquiterpenes that may formally be derived from humulene, a monocyclic C-15 hydrocarbon.[1] The humulene-derived sesquiterpenes comprise seventeen different skeletal types. The occurrence of humulene is widespread in plants and a number of humulene derived compounds are found in fungi.[2] It has been proposed that biosynthesis of humulene proceeds *via* primary cyclisation between C-1 and the distal, C-10–C-11 double bond of farnesyl diphosphate.[3] Recently, γ-humulene synthase has been cloned from *Abies grandis*.[4] Insight into illudin biosynthesis has been gained through a number of labelling studies of the illudane, illudin M, which is produced by *Omphalotus olearius* (formerly *Clitocybe illudens*). The original biogenetic proposal involved protonation of humulene to initiate further cyclisation and rearrangement to generate the illudane skeleton. Incorporation of ^{14}C label from [2-$^{3}H_2$, 2-^{14}C]mevalonate indicated that three molecules of mevalonic acid are incorporated into illudin M.[5] One labelled mevalonoid hydrogen from [4(*R*)-4-^{3}H]mevalonate is incorporated into illudin M.[5] A feeding experiment using [5-^{3}H]mevalonate indicated that the proton attached to C-6 of illudin M arises from the starter isoprene unit of farnesyl diphosphate (C-1 of farnesyl diphosphate), which is derived from the 5-*pro*-*R*-position of mevalonate.[6] This evidence is in agreement with the biosynthesis of illudins, *via* a humulene intermediate. A suggested modification of this proposed mechanism of cyclisation included the formation of an illudene skeleton, possibly formed by the non-concerted cyclisation of humulene.[5] This could account for the labelling of the cyclopropane region of illudin M by only one ^{3}H from [2-$^{3}H_2$]-mevalonate.[5] Another feeding study involving [2-$^{3}H_2$, 2-^{14}C]-mevalonate supported this observation and a non-stereospecific loss of hydrogen at a stage after the formation of farnesyl diphosphate was proposed to account for this phenomenon.[6] Feeding studies using [1,2-$^{13}C_2$]acetate showed that C-3 and C-11 of illudin M arose from carbons of an intact acetate unit, which indicated the stereochemistry of the folding of farnesyl diphosphate in the cyclopropane portion of the molecule during biosynthesis.[7] The loss of one mevalonoid proton from C-12 in the cyclopropane region of illudin M was observed again by ^{2}H NMR spectroscopy of illudin M arising from incorporation of [2-$^{2}H_3$]acetate and [5-$^{2}H_2$, 5-^{13}C]mevalonate.[8]

A new mechanism of illudin biosynthesis involving a protoilludyl-type intermediate was proposed by Cane and Nachbar, [9] following their studies showing the involvement of a protoilludyl cation in the fomannosin biosynthesis. The production of illudol, a fomannosin-type metabolite, by the Basidiomycete *Clitocybe illudens* (now *O. olearius*) led to the conclusion that a protoilludyl-type intermediate may also exist in the synthesis of illudin S and illudin M. All results of feeding experiments involving ^{13}C and ^{2}H labelled acetates and mevalonate are in agreement with cyclisation of illudin M *via* a protoilludene intermediate, following the formation of a humulene cation.[8] Fomajorin D, an isocoumarin-type sesquiterpene, produced by the related Basidiomycete, *Fomes annosus*, also exhibits ^{13}C acetate labelling patterns that are consistent with the cyclisation of farnesyl diphosphate *via* a protoilludyl cation or its equivalent.[10] The hydrocarbon, Δ-6-protoilludene has been isolated as a natural product from the fomannosin-producing Basidiomycete, *Fomitopsis insularis*.[11] The ability of *O. olearius* to convert synthetic ^{2}H labelled Δ-6-protoilludene to illudin M and illudin S suggests that this hydrocarbon is an intermediate in the illudin biosynthesis.[12]

During a survey of secondary metabolite production by *Omphalotus nidiformis*, a fomannosane-type sesquiterpene, illudosin **1**, was isolated as a major metabolite in liquid shake culture.[13] This provided an opportunity to study a more reduced illudin metabolite than the illudane-type sesquiterpene, illudin M **2**, to gain further insight into illudin biosynthesis. This investigation reports the incorporation of ^{13}C and ^{2}H from a range of labelled acetates into illudosin (and illudin M), followed by ^{13}C NMR spectroscopy.

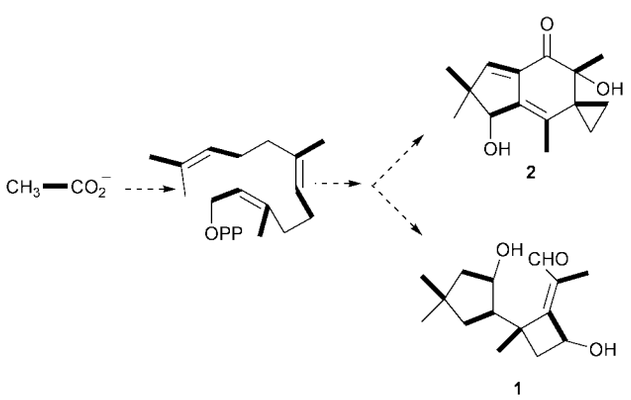
Results and discussion

Incorporation of ^{13}C and ^{2}H labels into illudosin and illudin M was observed in labelling experiments using [1-^{13}C]acetate, [2-^{13}C]acetate, [1,2-$^{13}C_2$]acetate and [1-^{13}C, 2-$^{2}H_3$]acetate (Tables 1 and 2). Prior to the application of labelling studies, the ^{1}H and ^{13}C NMR signals were assigned for unlabelled illudosin and illudin M by two-dimensional NMR experiments, and are in agreement with literature data.[8,14] Patterns of incorporation of ^{13}C labelled acetates into illudosin are shown in Scheme 1. ^{13}C Acetate labelling patterns of illudin M are in agreement with literature data,[6,7] including the observation of induced coupling

Table 1 Labelling of illudosin **1** by ^{2}H- and ^{13}C-labelled substrates

Carbon	δ_C (ppm)	Assignment	^{13}C–^{13}C J^c/Hz	Coupled ^{13}C nucleus	$\delta_C{}^d$ (ppm)	^{13}C resonance^e (%)	Coupled functional groups
1[a]	10.4	CH_3	43.4	2	10.44	100	—
2[b]	134.2	qC	43.4	1	134.15	78	CH_3
					134.12	11	CH_2D
					134.08	7	CHD_2
					134.05	4	CD_3
3[a]	191.8	CH=O	—	—	191.84	100	—
4[a]	172.4	qC	36.5	5	172.36	100	—
5[b]	67.4	CHOH	36.5	4	67.36	95	CH_2
					67.31	2	CHD
					67.27	3	CD_2
6[a]	39.8	CH_2	—	—	39.84	100	—
7[b]	45.8	qC	36.1	8	45.77	84	CH_3
					45.71	9	CH_2D
					45.64	7	CHD_2
8[a]	30.0	CH_3	36.1	7	29.99	100	—
9[a]	56.5	CH	34.0	10	56.52	100	—
10[b]	43.8	CH_2	34.0	9	43.82	88	CH
					43.78	12	CD
11[b]	36.7	qC	35.3	15	36.66	64	CH_3
					36.59	14	CH_2D
					36.52	12	CHD_2
					36.44	10	CD_3
12[a]	51.7	CH_2	36.7	13	51.68	100	—
13[b]	75.9	CHOH	36.7	12	75.88	91	CH_2
					75.82	9	CHD
14[b]	30.9	CH_3	—	—	30.91	100	—
15[a]	29.8	CH_3	35.3	11	29.81	100	—

[a] Labelled by [1-^{13}C]acetate feed. [b] Labelled by [2-^{13}C]acetate feed. [c] From ^{13}C NMR spectrum of illudosin labelled by [1,2-$^{13}C_2$]acetate. [d] From [1-^{13}C, 2-$^{2}H_3$]acetate feed. [e] Each ^{13}C signal is expressed as a percentage of the total area of the main ^{13}C resonance and the corresponding β-shifted ^{13}C resonances.

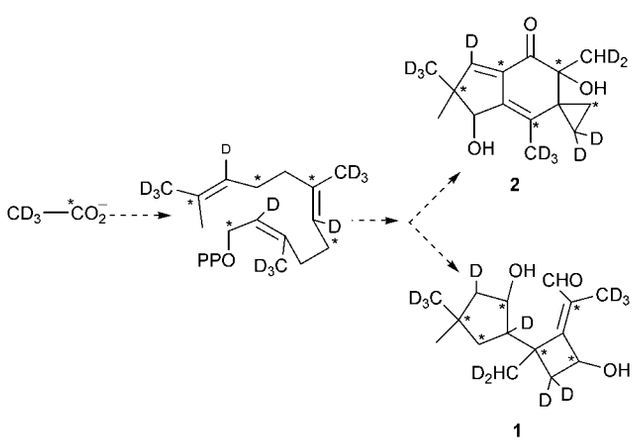


Scheme 1 Biosynthetic incorporation of [1,2-$^{13}C]_2$acetate into illudosin **1** and illudin M **2**.

between C-6 and C-7, carbon atoms that do not arise from incorporation of an intact acetate unit. These results are included here for comparison of the incorporation of labelled atoms into illudosin.

Sites of ^{2}H labelling were determined by indirect detection of the deuterium using ^{13}C NMR spectroscopy. ^{13}C NMR spectroscopy has much greater resolution than ^{2}H NMR spectroscopy and provides discrimination between various labelled sites. However, quantitative indirect detection of deuterium requires that the deuterium atom be located β† to a reporter ^{13}C nucleus. This arrangement results in the observation of incrementally upfield-shifted resonances that arise from the α-^{13}C nucleus. The presence of one, two, or three deuterium atoms attached to a carbon nucleus gives rise to one, two, or three upfield-shifted resonances, relative to the main α-^{13}C resonance.[15] Deuterium atoms directly attached to a ^{13}C nucleus also give rise to upfield-shifts of the α-^{13}C resonances and may show deuterium coupling. However, quantitative measurements are more difficult to obtain because the signal-to-noise ratio is reduced by poor relaxation, signal multiplicity, and loss of the nuclear Overhauser effect.[15]

Observation of deuterium labelling patterns of illudosin, which is more reduced than illudin M, has provided more information about the mechanism of cyclisation of farnesyl diphosphate. The deduced overall pattern of incorporation of deuterium labels into illudosin and illudin M are shown in Scheme 2. Illudosin labelled by [1-^{13}C–2-$^{2}H_3$]acetate exhibited



Scheme 2 Biosynthetic incorporation of [1-^{13}C,2-D_3]acetate into illudosin **1** and illudin M **2**. D denotes deuterium and * denotes ^{13}C.

upfield β-shifted resonances associated with carbons 5, 7, 10, 11, and 13. A sharp singlet and two smaller upfield-shifted resonances were observed for C-2 indicating the presence of molecules containing zero, one, or two deuterium atoms

† β refers to deuterons located one carbon from the ^{13}C reporter nucleus, *i.e.*, ^{2}H–^{12}C–^{13}C, while α refers to deuterons attached directly to the ^{13}C, *i.e.*, ^{2}H–^{13}C.

Table 2 Labelling of illudin M **2** by ^{2}H- and ^{13}C-labelled substrates

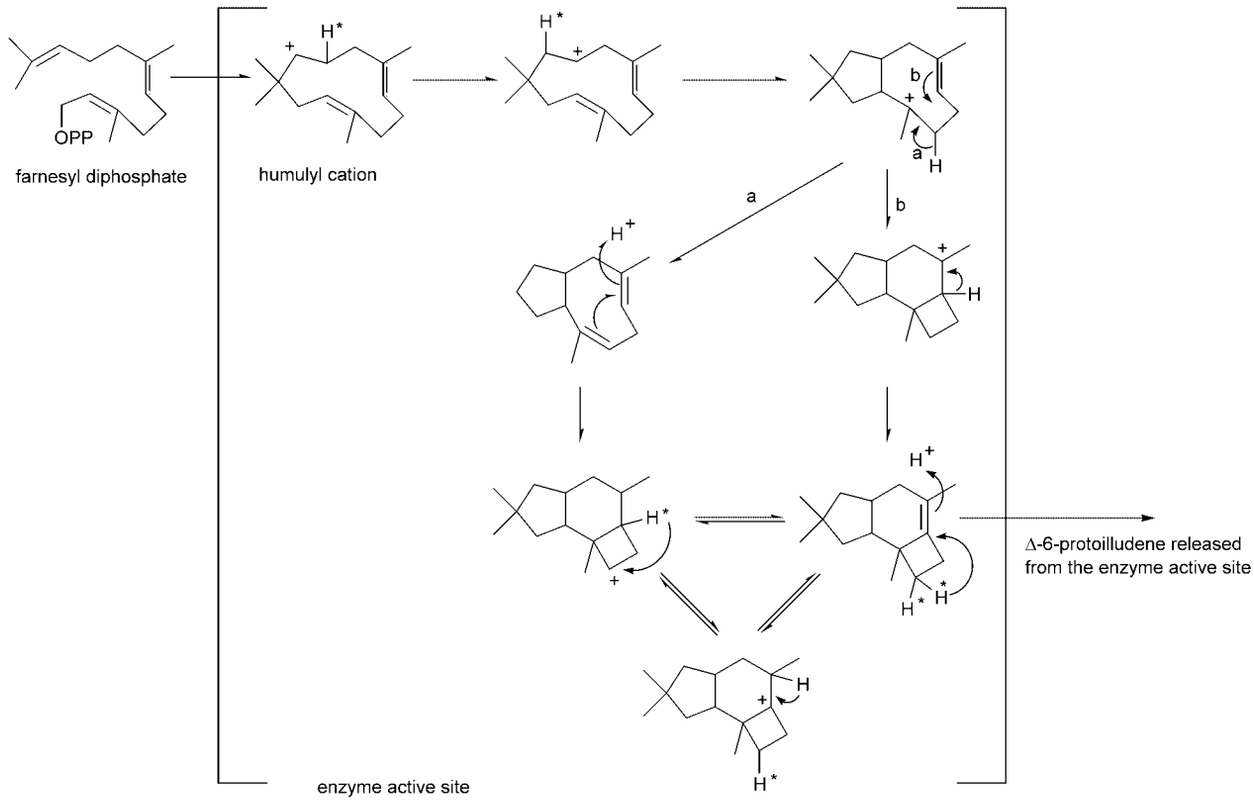
Carbon	δ_C (ppm)	Assignment	^{13}C–^{13}C J^c/Hz	Coupled ^{13}C nucleus	$\delta_C{}^d$ (ppm)	^{13}C resonance^f (%)	Coupled functional groups
1[b]	200.5	C=O	38.2	10	200.52	100	—
2[a]	76.0	C–OH	14.6	11	75.94	55	CH_3
					75.90	23	CH_2D
					75.87	23	CHD_2
3[b]	31.6	qC	44.5	13	31.61	100	—
4[a]	134.5	qC	47.0	6	134.47	51	CH_3
					134.4[e]	17	CH_2D
					134.4[e]	17	CHD_2
					134.4[e]	15	CD_3
5[b]	138.7	qC			138.73	100	—
6[a]	78.9	CH–OH	47.0	5	78.88	100	—
7[a]	49.0	qC	35.0	15	49.04	67	CH_3
					48.98	11	CH_2D
					48.91	14	CHD_2
					48.84	8	CD_3
8[b]	146.6	CH	68.0	9	146.62	100	—
9[a]	132.9	qC	68.0	8	132.94	83	CH
					132.83	17	CD
10[b]	24.8	CH_3	38.2	2	24.79	100	—
					5.93		
					5.82		
11[a]	5.8	CH_2	14.6	3	5.73	83	CH_2
						11	CHD
						6	CD_2
12[b]	8.6	CH_2	—	—	8.57	100	—
13[b]	14.2	CH_3	44.5	4	14.20	100	—
14[b]	20.5	CH_3	—	—	20.49	100	—
15[b]	27.3	CH_3	35.0	7	27.32	100	—

[a] Labelled by [1-^{13}C]acetate feed. [b] Labelled by [2-^{13}C]acetate feed. [c] From ^{13}C NMR spectrum of illudin M labelled by [1,2-$^{13}C_2$]acetate. [d] From [1-^{13}C, 2-$^{2}H_3$]acetate feed. [e] Unresolved multiplet—accurate chemical shifts not recorded. [f] Each ^{13}C signal is expressed as a percentage of the total area of the main ^{13}C resonance and the corresponding β-shifted ^{13}C resonances.

attached to C-1. A sharp singlet was observed for C-11 and three weaker signals were also observed upfield of the main signal, arising from molecules containing one, two, or three deuterium atoms attached to C-15. The [1,2-$^{13}C_2$]acetate feeding experiment indicated that C-15 and C-11 arose from incorporation of an intact acetate unit, which is in agreement with a literature report for illudin M.[9] Similarly, multiple resonances located upfield of the major C-7 resonance indicated the presence of up to three deuterium atoms attached to C-8. However, a broad multiplet was observed, rather than distinct peaks, due to the small β-shifts. C-10 exhibited one upfield-shifted signal associated with the main resonance, which is due to incorporation of one deuterium atom attached to C-9. The β-shifted resonance observed for C-13 established the retention of one deuterium atom at C-12.

The resonance at C-5 exhibited two upfield-shifted ^{13}C resonances that were weak compared with the main resonance. This was totally unexpected as the deuterium originally at C-6 of farnesyl diphosphate would have been predicted to be lost in forming the illudanes and illudosin. These may be accounted for by the intramolecular transfer of deuterium atoms from the carbon that becomes C-4 in illudosin to C-6. However, the [1,2-$^{13}C_2$]acetate feeding results clearly indicate that the C-4–C-5 bond arises from incorporation of an intact acetate unit. The deuterium atoms of the original methyl carbon in the intact acetate unit have been lost giving the quaternary C-4. Hence, the two deuterium atoms must be attached to C-6, β to the reporter ^{13}C nucleus at C-5. Similarly for illudin M, derived from [1-^{13}C, 2-$^{2}H_3$]acetate, rather weak resonances were observed 0.11 and 0.20 ppm upfield of the main signal arising from C-11 (equivalent to C-5 of illudosin), indicating the presence of two deuterium atoms located at C-12 (C-6 of illudosin). More labelling of both faces of the cyclobutane ring was observed in a small percentage of molecules. A possible mechanism involves a series of carbocation rearrangements within the sheltered active site of the enzyme (Scheme 3). A deuteron must be lost from C-6 of illudosin (C-12 illudin M), with some of it being delivered to the other face of the molecule, while these complex rearrangements are taking place. The involvement of the C-4 position of farnesyl diphosphate, that becomes C-6 in illudosin and C-12 in illudin M, has previously been suggested because of the loss of at least one tritium label in experiments involving incorporation of [2-$^{3}H_2$]mevalonate into illudin M.[6] Incorporation of [2*R*-^{3}H]- and [2*S*-^{3}H]mevalonate showed the loss to be non-stereoselective.[6] The loss of one mevalonoid proton from C-12 in the cyclopropane region of illudin M was observed again by ^{2}H NMR spectroscopy of illudin M arising from incorporation of [2-$^{2}H_3$]acetate and [5-$^{2}H_2$, 5-^{13}C]-mevalonate.[8] However, in each of these cases, the total amount of label attached to C-12 was determined without defining the stereochemistry of the incorporated label at C-12. Chemical shift values obtained in ^{2}H NMR spectra are similar to those of the equivalent protons observed in ^{1}H NMR spectra, so positions of deuteration are deduced by comparison to the corresponding ^{1}H NMR spectrum. However, the scale, in Hz, of a deuterium spectrum is only 15 percent of that of a proton NMR spectrum and spectral crowding is often observed. The relaxation of deuterium is dominated by a quadrupole exchange mechanism that results in deuterium linewidths being twenty times more sensitive to slow tumbling than those of protons;[16] as a result, peaks are often poorly resolved due to large linewidths. This may account for the report of resonances which integrate as a single deuterium at C-12 of illudin M,[8] rather than the isotopes being located on both faces of the cyclopropane ring.

It is possible to justify the presence of one deuterium attached to C-6 in illudosin if farnesyl diphosphate cyclises in a different manner than previously predicted,[6] with an intramolecular transfer of the 'C-4' deuterium to the C-6 position. However, this does not account for some molecules having two deuterium atoms attached to C-6. Further explanation involves consideration of the much lower amount of deuterium which is



Scheme 3 Proposed mechanism of cyclisation of farnesyl diphosphate.

incorporated at C-6. In both the ^{2}H and ^{13}C deuterium-shifted NMR spectra the incorporation at C-6 is considerably less than elsewhere. If a hydride migration occurs from the position that becomes C-4 in illudosin, to C-6, then a mechanism is needed to lose some of the deuterium at C-6 and to re-deuterate at the other C-6 position, *i.e.*, α→β.‡ One explanation is that this exchange is possible at the active site of the enzyme, where a series of carbocations are involved in a cyclisation–rearrangement. This is reinforced by considering what is known in the literature about active sites of terpene cyclases. The crystal structures of tobacco 5-*epi*-aristolochene synthase (TEAS) and pentalenene synthase both indicate the presence of Mg^{2+} coordination sites located in the enzyme active sites. Two Mg^{2+} ions are coordinated on opposite sides of the entrance to the active site pocket of TEAS, constituting a diphosphate binding site.[17] Pentalenene synthase possesses an aspartate rich, metal binding site which is thought to facilitate diphosphate departure in the first step of the cyclisation reaction by coordination of diphosphate to Mg^{2+} and bonding interactions with nearby basic residues.[18] Approximately 80% of the substrate surface area is buried in the pentalenene synthase–substrate complex, which helps to prevent premature quenching of the carbocation intermediates by solvent. It has been demonstrated that the bottom of the active site cavity is predominantly hydrophobic in nature and the overall shape of the cavity forms a template for the binding of farnesyl diphosphate in the correct conformation for cyclisation to pentalenene.[18] Similarly, Starks and co-workers[17] observed a closed solvent-inaccessible active site pocket in TEAS when the substrate was bound, which was consistent with the need to bind a hydrophobic substrate and to protect reactive carbocation intermediates from attack by water. They have shown that a 'J-K' loop of TEAS becomes ordered, forming a lid that clamps down over the active site entrance in the presence of a farnesyl diphosphate analogue, farnesyl hydroxyphosphonate, and that this clamp forms an extended aromatic box deep within the active site pocket.[17] A catalytic mechanism was proposed that involved the generation of a diphosphate ion, that has its additional negative charge offset by interactions with Mg^{2+} ions and arginine residues. This concentrated region of positive charge is thought to direct the diphosphate away from the hydrophobic pocket where a number of highly reactive carbocations are formed throughout the reaction. The cationic end of the farnesyl chain is directed into the active site and stabilised by dipolar interactions with amide, aromatic and hydroxy functional groups.[17] Sequence alignments between TEAS and other cloned plant cyclases suggest that all plant cyclases probably share similar three dimensional structures.[17] In fact, several chimeras of TEAS and vetispiradiene synthase (HVS), a sesquiterpene cyclase from *Hyoscamus muticus* that catalyses cyclisation of farnesyl diphosphate to vetispiradiene,[19] have been shown to produce mixtures of 5-*epi*-aristolochene and vetispiradiene.[20] These multi-product chimeric enzymes, in which the active site topology is influenced by a combination of interactions from the two wild-type enzymes, still catalyse terpene cyclisation, but they have lost the ability to select a single conformation of the eudesmane intermediate that would give rise to a single product.[20] The kinetic mechanisms of these terpene cyclases involve the rapid formation of an enzyme substrate complex followed by conversion of enzyme bound farnesyl diphosphate to an enzyme bound hydrocarbon product, *via* a series of carbocation intermediates, followed by the slow and rate limiting release of the hydrocarbon from the enzyme.[21] Consideration of the sizes of the active sites of the two sesquiterpene cyclases, whose three dimensional crystal structures are known, would seem to indicate that drastic reorientation of the reaction intermediates is unlikely. That is, it is very unlikely that a specific amino acid side chain or amide bond at the active site could accept a deuteron from the substrate and deliver it to the opposite face of the molecule. The process that we have depicted in Scheme 3 implies a non-stereospecific loss of a proton from the position that becomes C-6 in illudosin (C-12 in illudin M). This happens in the confines of the active site, away from direct contact with bulk water, so there is an opportunity

‡ α and β refer to the two faces of the protoilludene intermediate.

for non-specific reprotonation (or deuteration) to occur, which would give the appearance of an intramolecular rearrangement.§ The involvement of isotope effects would also influence these results, as the cleavage of the C–^{2}H bond would be expected to be considerably slower than the cleavage of the C–^{1}H bond. The detailed study of these effects will be dependent upon purification of the cyclase and/or production of an active recombinant enzyme by overexpression of a suitable clone.

The observed patterns of incorporation of ^{13}C and ^{2}H labels into illudosin are consistent with the labelling of the isocoumarin, fomajorin D.[10] In particular, the C-4–C-5 bond arises from an intact acetate unit and there has been no unusual rearrangement of the carbon skeleton. The labelling of C-12 and C-9 with deuterium establishes that C-10, and not C-12 of illudosin arises from the 1-position of farnesyl diphosphate. The intensity of the shifted resonance in both cases is about 10% of the main signal. The incorporation of deuterium label into the methyl groups C-1, C-8, and C-15 is larger. The loss of deuterium probably occurs at the stage of acetoacetyl CoA formation, before HMGCoA is synthesised.

Incorporation of ^{13}C labels into illudin M was consistent with previous literature reports[5,7,8] and with the labelling pattern we observed for illudosin. However, illudin M is less protonated than illudosin so fewer deuterium atoms are incorporated into illudin M in the [1-^{13}C, 2-$^{2}H_3$]acetate feeding experiment. C-2, C-4, C-7, C-9, and C-11 exhibited associated upfield shifted resonances. A sharp singlet and two smaller upfield-shifted singlets were observed for C-2 indicating the incorporation of two deuterium atoms attached to C-10. Three upfield shifted signals were associated with the main resonance of C-7, arising from molecules containing up to three deuterium atoms attached to C-15. Similarly, multiple resonances located upfield of the major C-4 resonance indicated the presence of up to three deuterium atoms attached to C-13. The β-shifted resonance associated with the resonance of C-9 (0.09 ppm upfield) established the retention of one deuterium atom at C-8. These results support a pathway in which intramolecular cyclisation of farnesyl diphosphate gives rise to a humulyl cation. Further cyclisation to a protoilludyl cation followed by ring contraction, hydroxylation, and oxidation reactions would yield illudin M and other illudane-type metabolites of *O. nidiformis*. Cleavage of the six membered ring of the protoilludyl intermediate, and subsequent hydroxylation and oxidation would give rise to the fomannosane-type sesquiterpene, illudosin.

Δ-6-Protoilludene has been isolated as a natural product from *Fomitopsis insularis*, a fungus producing the sesquiterpene, fomannosin[11] and synthetic deuterium-labelled Δ-6-protoilludene has been converted to illudin M and illudin S by *Omphalotus olearius*.[13] Our results are fully consistent with Δ-6-protoilludene being an intermediate, possibly enzyme bound, in the biosynthesis of the illudane sesquiterpenes.

Experimental

O. nidiformis (B. J. Rees, Culture No UNSW 057) was maintained on malt extract agar at 23 °C in the dark. The malt extract agar contained 20 g malt extract and 20 g agar per litre of water. The pH was adjusted to 6.5, prior to sterilisation. Seed cultures were prepared by inoculation of 300 ml liquid malt extract medium in 1 l baffled flasks with mycelia from two week old plate cultures. These cultures were incubated at 23 °C with orbital shaking (100 rpm) in the dark. After ten days growth, cultures were homogenised in a Waring-type blender for two bursts of one second duration. The resulting suspension was used as a 10% inoculum for 300 ml shake cultures, in 1 l baffled flasks. *O. nidiformis* shake cultures (2 × 300 ml) were employed for each labelling experiment. A total of 40 mg of labelled acetate ([1-^{13}C]-acetate, [2-^{13}C]-acetate, [1,2-$^{13}C_2$]acetate and [1-^{13}C, 2-$^{2}H_3$]acetate) was added to each culture in 10 mg aliquots at regular intervals between 46 and 76 h after inoculation. Cultures were harvested after 130 h growth and the culture broth was extracted with EtOAc (equal vol.). Solvent was removed by rotary evaporation at 30 °C. Illudosin and illudin M were obtained by preparative layer chromatography using silica gel G TLC plates (Merck Darmstadt). Each plate was developed twice using EtOAc–hexane (1 : 9 v/v) and metabolites were identified by comparison to authentic standards.

^{1}H NMR spectra were acquired at 500.13 MHz using a Bruker Avance Series 500 spectrometer. Spectra were recorded with a 90° pulse of typically 3.6 μs, 6600 Hz spectral width, 16 000 data points and a one second relaxation delay between pulses in spinning sample tubes at 25 °C. The primary reference was $CHCl_3$, which resonates at 7.26 ppm relative to Me_4Si. ^{13}C NMR spectra were acquired at 150.92 MHz using a Bruker Avance Series 600 spectrometer. 8000 to 25 000 scans were acquired depending on sample concentration. A spectral width of 34 000 Hz, a 4 μs pulse and a relaxation delay of 2 seconds were employed. Each ^{13}C signal of the spectra acquired for illudosin and illudin M labelled by [1-^{13}C, 2-$^{2}H_3$]acetate is expressed as a percentage of the total area of the main ^{13}C resonance and the corresponding β-shifted resonances.

Illudin M

δ_H (500 MHz; $CDCl_3$): 0.41 (m, 1H, H-12$_\alpha$), 0.84 (m, 1H, H-12$_\beta$), 0.96 (m, 1H, H-11$_\alpha$), 1.10 (s, 3H, H-15), 1.12 (m, 1H, H-11$_\beta$), 1.20 (s, 3H, H-14), 1.35 (s, 3H, H-10), 1.68 (s, 3H, H-13), 3.48 (br s, 1H, OH), 3.57 (br s, 1H, OH), 4.39 (s, 1H, H-6) and 6.53 (s, 1H, H-8).

Illudosin

δ_H (500 MHz; $CDCl_3$): 1.00 (s, 3H, H-14), 1.08 (s, 3H, H-15), 1.15 (t, 1H, H-10$_\alpha$), 1.49 (m, 1H, H-12$_\alpha$), 1.53 (m, 1H, H-10$_\beta$), 1.59 (s, 3H, H-8), 1.72 (s, 3H, H-1), 1.75 (d, 1H, H-12$_\beta$), 1.76 (m, 2H, H-6$_\alpha$), 2.28 (m, 1H, H-9), 2.52 (m, 1H, H-6$_\beta$), 3.95 (br m, 1H, H-13), 4.72 (br m, 1H, H-5), 9.90 (s, 1H, H-3).

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