Use of Deuterium as a Tracer with ^{13}C Nuclear Magnetic Resonance Spectroscopy in Following Deuteride Migration in Terpenoid Biosynthesis: Mechanism of Geranylgeranyl Pyrophosphate Cyclisation in Fusicoccin Biosynthesis

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Summary Three of the four (4*R*) mevalonoid atoms retained in fusicoccin (**1a**) have been located at C-7, C-15, and C-23 by ^{13}C n.m.r. spectroscopy, following the incorporation of [3-^{13}C, 4-$^{2}H_2$]mevalonolactone; a 1,2-deuteride shift is demonstrated in the formation of the bicyclic intermediate (**3**), whilst two consecutive 1,2-deuteride shifts are established during the further cyclisation of (**3**) to (**1a**).

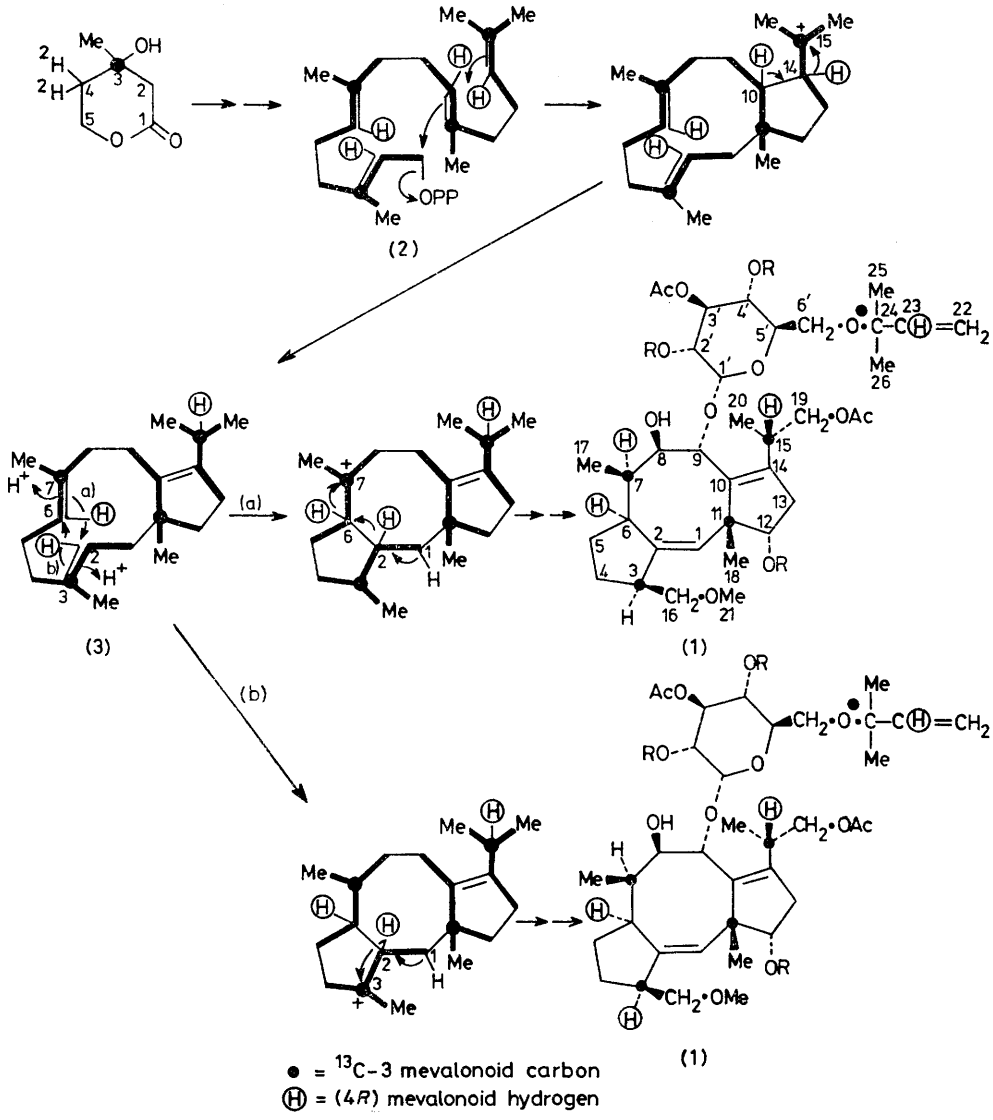
THE use of deuterium as a tracer, in conjunction with ^{13}C n.m.r. spectroscopy, in exploring mechanism in biosynthesis and chemical studies has found only limited use so far.[1] In biosynthetic studies the technique has been confined to investigating changes in the integrity of ^{13}C–^{2}H bonds in $^{13}C^{2}H_3$ groups of methionine and acetate during biological methylation and in polyketide synthesis, respectively. The principle of the approach relies on the longer relaxation time (T_1) of ^{13}C and the absence of a ^{1}H nuclear Overhauser effect in ^{13}C–^{2}H (*cf.* ^{13}C–^{1}H) resulting in marked suppression of the signal intensity of ^{13}C in the proton-noise-decoupled spectrum. Isotopic substitution of ^{13}C additionally results in an upfield shift of the ^{13}C signal by *ca.* 0·3 p.p.m. per ^{2}H atom,[2] whereas secondary isotopic substitution (*i.e.* ^{13}C–C–^{2}H) results in a smaller but detectable upfield shift of *ca.* 0·1 p.p.m. per ^{2}H atom.[3]

The use of deuterium tracers with ^{13}C n.m.r. spectroscopy in locating mevalonoid hydrogen atoms and in following hydrogen shifts in terpenoid biosynthesis has not been reported.

We report here the usefulness of the technique in elucidating the mechanism of geranylgeranyl pyrophosphate (**2**) cyclisation, in which two hydride shifts are demonstrated, during the biosynthesis of the fungal phytotoxin fusicoccin (**1a**). We have previously demonstrated that (**1a**) is a diterpenoid[4] and would therefore arise from the cyclisation of (**2**). The fifth and isolated isoprenoid residue attached to the sugar unit is probably appended at a late stage in the biosynthesis.[5] Four out of a possible five (4*R*) mevalonoid tritium atoms are incorporated into (**1a**).[6]† This

† In the present study [(4*R*)-4-^{3}H, 2-^{14}C]mevalonate (^{3}H: ^{14}C atom ratio 5:5) was incorporated into (**1a**) which had a ^{3}H:^{14}C atom ratio of 3·94:5.

result, together with the labelling of C-3, C-7, C-11, C-15, and C-24 from [3-^{13}C]mevalonate,[6] suggested two possible modes of cyclisation of the bicyclic intermediate (**3**) [Scheme, (a) and (b)], which differ in the direction of C–C bond formation between C-2 and C-6. Route (a) requires either two 1,2-hydride shifts or one 1,3-hydride shift, while route (b) necessitates only one 1,2-hydride shift. Importantly the 1,2-hydride shifts from C-2 to C-3 and C-6 to C-7 involve migration of a (4*R*)-mevalonoid hydrogen within a single isoprenoid residue, whilst a 1,3-hydride shift from C-2 to C-7 [pathway (a)] would involve a (4*R*)-mevalonoid hydrogen transfer between discrete isoprenoid residues. In each case the hydrogen transfer takes place to a C-3 mevalonoid carbon atom. Similarly, in generating the bicyclic intermediate (**3**), double-bond formation could involve two 1,2-hydride shifts, in which one of the two (4*R*)-mevalonoid hydrogens migrates within an isoprenoid residue (C-14 to C-15) to a C-3 mevalonoid carbon or a less likely inter-isoprenoid 1,3-transfer of a (4*R*) mevalonoid hydrogen from C-10 to C-15.



(**1a**), R = H
(**1b**), R = OAc

SCHEME

These alternative mechanisms were evaluated by following the translocation of ^{2}H to enriched ^{13}C centres following the incorporation of [4-$^{2}H_2$, 3-^{13}C]-(3*RS*)mevalonolactone. The doubly labelled precursor was synthesised from sodium [1-^{13}C]acetate (90·3 atom % ^{13}C) and methyl [2-$^{2}H_3$] acetate (99·5% ^{2}H) essentially as described by Tanabe.[7] That the C-4 ^{2}H atoms in the doubly labelled mevalonolactone had not exchanged (or scrambled) for ^{1}H during the synthesis was evident from the n.m.r. spectra of the precursor: ^{1}H n.m.r. spectrum, δ 1·40 [3H, d, J 4·5 Hz (CH_3–^{13}C)

superimposed on a central singlet (*ca.* 10%, CH_3–^{12}C)], 2·55 (2H, m, H-2), 2·90 (1H, br. s, OH), and 4·45 (2H, m, H-5) (no signal was observed for H-4 at δ 1·9); ^{13}C n.m.r. spectrum, δ 29·6 [d, *J* 40 Hz, *ca.* 10%, C-3′), 35·8 (collapsed m, C-4), 44·6 (d, *J* 36·5 Hz, *ca.* 10%, C-2), 66·2 (d, *J* 2·1 Hz, *ca.* 10%, C-5), 68·1 (br. s, *ca.* 90%, (C-3), and 171·1 (s, *ca.* 10%, C-1) p.p.m.

Enriched (**1a**) was isolated from cultures (8 × 100 ml) of *Fusicoccum amygdali*[6] supplemented with [4-$^2H_2$, 3-^{13}C]-3(*RS*)mevalonolactone (392 mg, 90·3 atom % ^{13}C, 99·5% ^2H) containing a small amount of [2-^{14}C]-(3*RS*)mevalonolactone (1·89 × 10^7 disint. min^{-1}), and converted into its triacetate derivative (**1b**) (203 mg, 483 disint. min^{-1} mg^{-1}) for spectroscopic analysis. Assuming an equal distribution of ^{14}C between the five isoprenoid residues, the degree of radioactive label in (**1b**) indicated that C-3, C-7, C-11, C-15, and C-24 had each been enriched with ^{13}C by *ca.* 110% in good agreement with the observed enhancement of the C-3, C-11, and C-24 signals in the spectrum of (**1b**). The signals in the proton-noise-decoupled natural-abundance spectrum of (**1b**) and in the spectrum of the ^2H–^{13}C enriched (**1b**), which were recorded under identical instrument conditions, were measured by integration. The signal intensities were normalised on to C-21, which is not labelled from [3-^{13}C]-(3*RS*)mevalonolactone.[5] The results are collected in the Table.

TABLE. ^{13}C Enrichment of fusicoccin triacetate (**1b**)

Assignment[a]	Chemical shift[b]	Enrichment factor[c]
C-3	47·85	1·92
C-7	40·68	0·97
C-11	54·74	1·85
C-15	32·64	1·01
C24 (–CMe_2CH=)	74·85	1·97
C24 (–CMe_2C^2H=)	74·77	
Remaining signals		0·98 ± 0·07

[a] From ref. 5. [b] ^{13}C N.m.r. spectra were recorded in CCl_4 on a Varian XL-100-12 n.m.r. spectrometer at 25·2 MHz; signals are reported in p.p.m. to low field of Me_4Si. [c] Peak integral: (enriched sample normalised w.r.t. C-21)/(natural abundance sample normalised w.r.t. C-21).

The intensities of the signals due to C-7 and C-15 were markedly suppressed in comparison with the signal intensities of C-3, C-11, and C-24, clearly owing to the presence of one ^2H atom at each of the former positions (unfortunately the intensities of the ^{13}C–^2H triplets for C-7 and C-15 were too weak to be observed). This result rules out pathway (b) and supports, in pathway (a), two consecutive 1,2-hydride shifts, rather than a 1,3-hydride shift. A 1,3-^2H shift would involve ^2H transfer between C-2 and C-7 across separate isoprenoid residues. Since the [4-$^2H_2$, 3-^{13}C]mevalonic acid would enter the cellular pool of unlabelled mevalonic acid, the probability that more than one isoprenoid residue in the polyprenyl pyrophosphate chain would be enriched in ^2H and ^{13}C is negligible. Thus, a 1,3-shift of ^2H would place the transferred ^2H on ^{12}C rather than an enriched ^{13}C centre. The reduced intensity of the C-15 signal also supported the mechanism as shown, rather than a 1,3-hydride shift, from C-10 to C-15 followed by double bond formation. Thus the carbocyclic ring system of (**1a**) is formed by a different mechanism to that observed in the formation of the structurally similar ophiobolin fungal metabolites.[8]

The signal due to C-24 appeared as two lines separated by 2 Hz, in accord with chemical shift differences observed for ^{13}C–C–H → ^{13}C–C–^2H. Thus, the upfield signal is due to molecules of (**1b**) carrying ^2H at C-23 and ^{13}C at C-24 as expected, whilst the line to low field represents C-24 in unenriched molecules.

These observations emphasise the usefulness of using carefully chosen, specifically ^2H and ^{13}C labelled mevalonates in following ^2H shifts and locating ^2H atoms two bonds distant from a ^{13}C nucleus in terpenoid biosynthesis.

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