Fusicoccin. Part 6.[1] Biosynthesis of Fusicoccin from [3-^{13}C]- and (4*R*)-[4-$^{3}H_1$]-Mevalonic Acid

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[3-^{13}C]Mevalonic acid lactone has been synthesised from [1-^{13}C]acetate and selectively incorporated into positions 3,7,11,15, and 24 of fusicoccin (1a). Four out of a possible five 4-*pro-R* mevalonoid hydrogen atoms were retained in fusicoccin and fusicoccin J, and three were retained in fusicoccin H. The results are consistent with the cyclisation of a polyprenyl pyrophosphate precursor in a manner different to that previously observed in the biosynthesis of the ophiobolins.

FUSICOCCIN (Ia)[2] is the predominant component of a family of structurally related phytotoxic metabolites [3-6] produced when the plant pathogenic fungus *Fusicoccum amygdali* Del. is grown in submerged culture. With the exception of fusicoccin H[5] (IIa), all the fusicoccin glycosides isolated to date carry an isoprenoid residue at C-6′ of the sugar. The former observation has strongly suggested [5] that the fusicoccins are diterpenoids (*cf.* the cotylenins) and not rearranged sesterterpenoids. The aglycone of (1a) has the same carbocyclic skeleton as the ophiobolins [7] [*e.g.* ophiobolin F [8] (VII)], cotylenins [9] [*e.g.* cotylenin E [9b] (III)], and ceroplastanes [10] [*e.g.* ceroplasteric acid [10a] (IV)]. With the exception of the

[1] Part 5, K. D. Barrow, R. B. Jones, P. W. Pemberton, and L. Phillips, *J.C.S. Perkin I*, 1975, 1405.

[2] A. Ballio, E. B. Chain, P. de Leo, B. F. Erlanger, M. Mauri, and A. Tonolo, *Nature*, 1964, **203**, 297; A. Ballio, M. Bufani, C. G. Casinovi, S. Cerrini, W. Fedeli, R. Pellicciari, B. Santurbano, and A. Vaciago, *Experientia*, 1968, **24**, 631; K. D. Barrow, D. H. R. Barton, E. B. Chain, U. F. W. Ohnsorge, and R. Thomas, *Chem. Comm.*, 1968, 1198; E. Hough, M. B. Hursthouse, S. Neidle, and D. Rogers, *Chem. Comm.*, 1968, 1197; K. D. Barrow, D. H. R. Barton, E. B. Chain, U. F. W. Ohnsorge, and R. Thomas, *J. Chem. Soc.* (*C*), 1971, 1265; K. D. Barrow, D. H. R. Barton, E. B. Chain, C. Conlay, T. C. Smale, R. Thomas, and E. S. Waight, *J. Chem. Soc.* (*C*), 1971, 1259.

[3] A. Ballio, C. G. Casinovi, G. Randazzo, and C. Rossi, *Experientia*, 1970, **26**, 349.

[4] A. Ballio, C. G. Casinovi, M. Framondino, G. Grandolini, F. Merichini, G. Randazzo, and C. Rossi, *Experientia*, 1972, **28**, 126.

[5] (*a*) K. D. Barrow, D. H. R. Barton, E. B. Chain, U. F. W. Ohnsorge, and R. P. Sharma, *J. Chem. Soc.* (*C*), 1973, 1590; (*b*) A. Ballio, C. G. Casinovi, M. Framondino, G. Grandolini, G. Randazzo, and C. Rossi, *Experientia*, 1972, **28**, 1150.

[6] K. D. Barrow, D. H. R. Barton, E. B. Chain, D. Bageenda-Kasujja, and G. Mellows, *J.C.S. Perkin I*, 1975, 877; A. Ballio, C. G. Casinovi, V. D'Alessio, G. Grandolini, G. Randazzo, and C. Rossi, *Experientia*, 1974, **30**, 844.

[7] K. Tsuda, S. Nozoe, M. Morisaki, K. Hirai, A. Itai, S. Okuda, L. Canonica, A. Fiecchi, M. Galli-Kienla, and A. Scala, *Tetrahedron Letters*, 1967, 3369, and references cited therein.

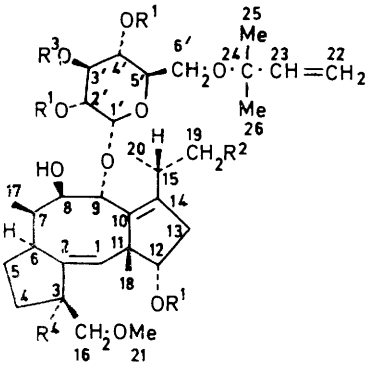
[8] S. Nozoe, M. Morisaki, K. Fukushima, and S. Okuda, *Tetrahedron Letters*, 1968, 4457.

[9] (*a*) T. Sassa, A. Takahama, and T. Shindo, *Agric. and Biol. Chem.* (*Japan*), 1975, **39**, 1729; (*b*) T. Sassa, M. Toyoshi, and T. Kitaguchi, *ibid.*, p. 1735; (*c*) T. Sassa and A. Takahama, *ibid.*, p. 2213.

[10] (*a*) Y. Iitaka, I. Watanabe, I. T. Harrison, and S. Harrison, *J. Amer. Chem. Soc.*, 1968, **90**, 1092; (*b*) T. Rios and L. Quijano, *Tetrahedron Letters*, 1969, 1317; (*c*) T. Rios, L. Quijano, and J. Calderon, *J.C.S. Chem. Comm.*, 1974, 728.

cotylenins, these metabolites are all enzymically constructed from a polyprenyl pyrophosphate containing five isoprenoid units, and are therefore sesterterpenoids.

The biosynthesis of the ophiobolins has been extensively studied by Canonica and his co-workers. During the cyclisation of geranylfarnesyl pyrophosphate (V) it has been shown that all the C-2 mevalonoid hydrogen atoms are retained [11] and that a 2-*pro-R* mevalonoid hydrogen undergoes an enzyme-mediated 1,5-shift from C-8 to C-15 of the ophiobolin precursor.[11,12] Also, no 4-*pro-R* mevalonoid hydrogen atoms were lost in the formation of ophiobolin C.[13] On the basis of this and



(I)

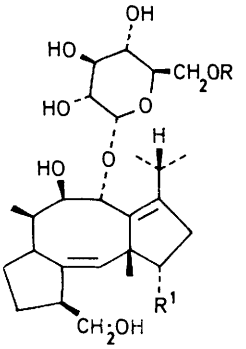
a; $R^1=R^4=H$, $R^2=OAc$, $R^3=Ac$

b; $R^1=R^3=Ac$, $R^2=OAc$, $R^4=H$

c; $R^1=R^3=R^4=H$, $R^2=H$

d; $R^1=R^2=R^3=R^4=H$

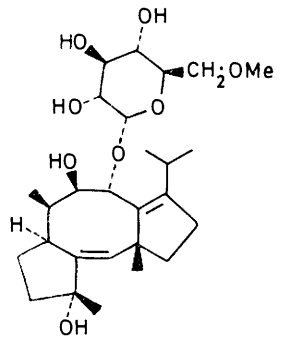
e; $R^1=R^2=R^3=H$, $R^4=OH$



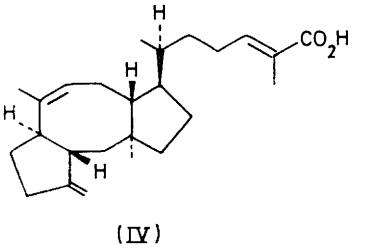
(II)

a; $R^1=R^2=H$

b; $R^1=CMe_2{\cdot}CH:CH_2$, $R^2=OH$



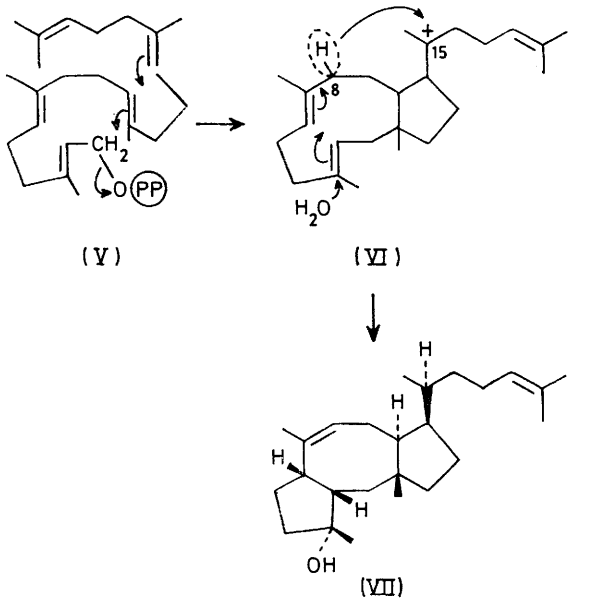
(III)



(IV)

other evidence the mode of cyclisation of the polyprenyl pyrophosphate precursor (V) depicted in Scheme 1 is well substantiated in ophiobolin biosynthesis. An analogous mechanism has been proposed for the formation of the ceroplastanes.[10c]

As well as carrying a substituted glucose residue at C-9, the aglycone of the fusicoccins differs from the



(V) (VI) (VII)

SCHEME 1

carbocyclic skeleton of the above terpenoids in two notable respects. With the exception of the recently characterised fusicoccin (Ie),[14] the fusicoccin family do not carry a tertiary hydroxy-substituent at C-3 and, with the exception of the cotylenins, the unsaturation pattern is different.

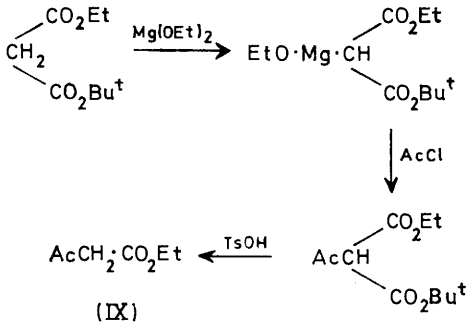
In furtherance of our biosynthetic studies, we now report the synthesis and incorporation of [3-^{13}C] mevalonic acid into fusicoccin, and present evidence which indicates a mechanism different from that observed in ophiobolin F (VII) biosynthesis, during the cyclisation of the polyprenyl pyrophosphate.

Synthesis of [3-^{13}C]*Mevalonolactone.*—The [3-^{13}C]mevalonic acid used was synthesised by a route, proceeding *via* ethyl [3-^{13}C]acetoacetate, developed by us for the preparation of multiply labelled mevalonic acid species for biosynthetic purposes. Although there are obvious shorter routes to [3-^{13}C]mevalonic acid (*e.g.* ref. 15), the method described here is adaptable and particularly useful for small-scale preparations.

Ethyl [3-^{13}C]*acetoacetate.* Although several methods for the synthesis of ethyl acetoacetate (IX) have been reported, none was satisfactory for our purposes because of either poor yields or the nature of the starting material. For example the standard Claisen condensation of ethyl acetate, besides requiring 2 mol of starting material per

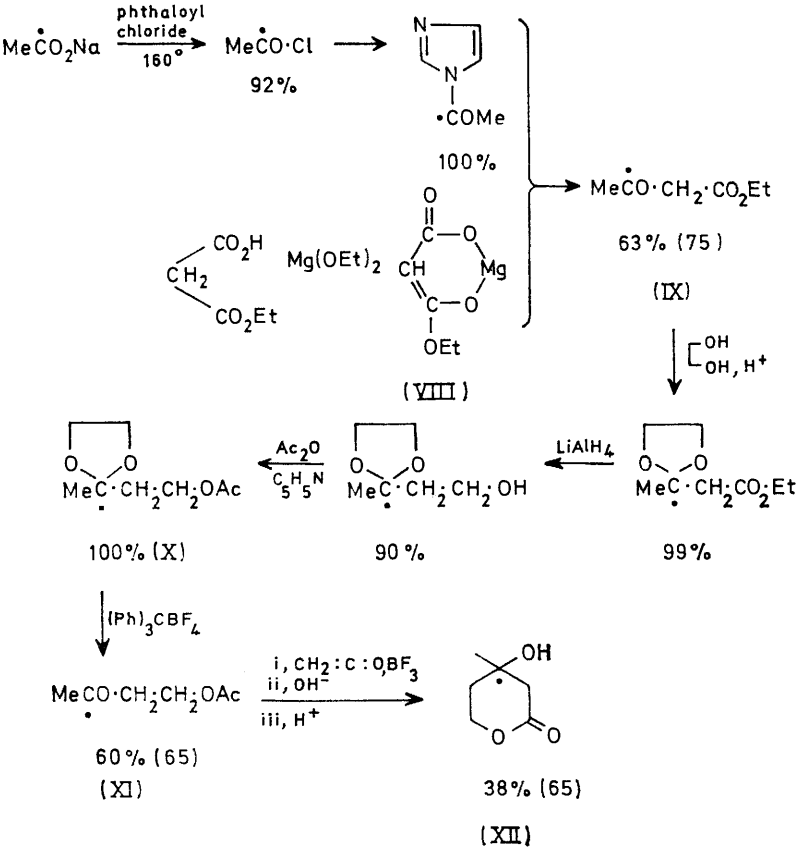
[11] L. Canonica, A. Fiecchi, M. Galli-Kienle, B. M. Ranzi, A. Scala, T. Salvatori, and E. Pella, *Tetrahedron Letters*, 1967, 3371.
[12] L. Canonica, A. Fiecchi, M. Galli-Kienle, B. M. Ranzi, and A Scala, *Tetrahedron Letters*, 1967, 4657.
[13] L. Canonica, F. Fiecchi, M. Galli-Kienle, B. M. Ranzi, and A Scala, *Tetrahedron Letters*, 1968, 275.
[14] A. Ballio, C. G. Casinovi, G. Grandolini, F. Marta, G. Randazzo, and C. Rossi, to be published (quoted in ref. 26).
[15] J. A. Lawson, W. T. Colwell, J. I. DeGraw, R. H. Peters, R. L. Dehn, and M. Tanabe, *Synthesis*, 1975, 729.

mol of product, gives only 29% yield,[16] whereas a claim of 80% yield by modification of this procedure was calculated on the basis of sodium ethoxide used in the



SCHEME 2

reaction.[17] Although several good syntheses of β-oxo-esters have been reported,[18-20] there is no report of their application to ethyl acetoacetate. Initially we investigated an unpublished procedure based on the method of Breslow and Baurngarten,[18] kindly provided by Professor J. W. Cornforth (Scheme 2), which had given yields of up to 62%. However we could not obtain more than a 45% yield of (IX), in spite of considerable modification of reaction conditions. The required product was always contaminated with diethyl malonate and diethyl acetylmalonate, which could not be easily separated. The formation of diethyl acylmalonates in this type of reaction has been observed previously.[18] The procedure finally adopted was based on the method of Braum and Vikas [19] (Scheme 3). A consistent yield of up to 75% of the acetoacetate (IX) could be obtained if the ethanol formed in the generation of the magnesium complex (VIII) was removed prior to acylation and the acylating agent was *N*-acetylimidazole. The latter was conveniently prepared from acetyl chloride ([1-^{13}C]acetyl chloride in ^{13}C synthesis) and *N*-trimethylsilylimidazole. Attempts to prepare *N*-acetylimidazole directly by the reaction of acetyl chloride with imidazole, acetic acid, and *NN'*-carbonyldi-imidazole or acetic anhydride and imidazole resulted in lower yields. Ethyl [3-^{13}C]acetoacetate was prepared in good yield by the above procedure.

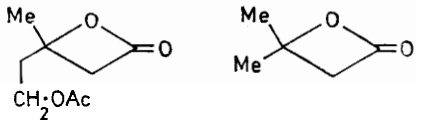


SCHEME 3 Figures in parentheses refer to yields obtained with unlabelled compounds

[16] J. K. H. Inglis and F. C. Roberts, *Org. Synth.*, Coll. Vol. I., 1941, p. 235.
[17] D. C. Roberts and S. M. McElvain, *J. Amer. Chem. Soc.*, 1937, **59**, 2007.
[18] D. S. Breslow and E. Bourngarten, *J. Amer. Chem. Soc.*, 1944, **66**, 1286.
[19] G. Braum and M. Vilkas, *Bull. Soc. chim. France*, 1964, 945.
[20] R. E. Ireland and J. A. Marshall, *J. Amer. Chem. Soc.*, 1959, **81**, 2907.

[3-^{13}C]-3-*Oxobutyl acetate* (XI). This was prepared in high yield from ethyl [3-^{13}C]acetoacetate essentially as described by Canonica and his co-workers,[21] but with the following modification. The deacetalisation of the ethylene acetal (X) was carried out rapidly with trityl tetrafluoroborate [22] in methylene chloride, thus avoiding the prolonged reaction time with 0.1N-sulphuric acid.[21]

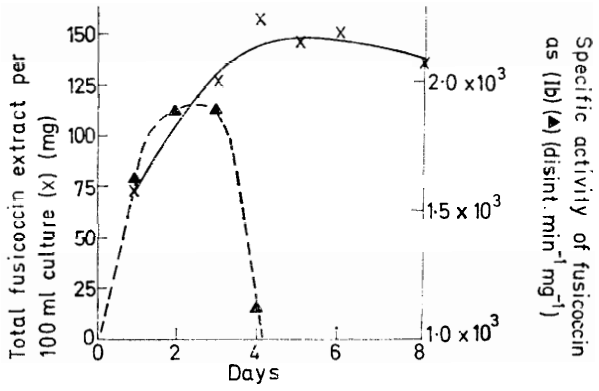
[3-^{13}C]*Mevalonolactone* (XII).—Conversion of the [3-^{13}C]-oxo-ester (XI) into the [3-^{13}C]-lactone (XII) was accomplished by Cornforth's keten procedure,[23] with some modification, in 38% yield, although in trial reactions yields of 60—65% were obtained consistently. The lower yields on a small scale (*cf.* 90% on large scale) resulted from the difficulty encountered in the separation of isovaleric acid (produced by the reaction of keten with acetone used in the generator). In an attempt to improve the yield of (XII), the intermediate β-lactone (XIII), m.p. 38—41°, $\nu_{max.}$ 1 822 and 1 742 cm^{-1}, was isolated and characterised. We anticipated that the β-lactones (XIII) and (XIV) would react with *NN*-di-isopropylhydrazine to produce crystalline hydrazides which might be more readily separated. Removal of the protecting group could then be accomplished by mild oxidation. Surprisingly (XIII) was unreactive towards *NN*-di-isopropyl- and *N*-isopropyl-hydrazine, and this approach was discontinued.



(XIII) (XIV)

After this synthesis of [3-^{13}C]mevalonic acid was completed, Ellison and Bhatnagar reported [24] the synthesis of ethyl 5-acetoxy-3-hydroxy-3-methylpentanoate, in high yield from ethyl lithioacetate and 3-oxobutyl acetate, which was converted into mevalonic acid by treatment with base in high yield. We have since coupled this procedure with our synthesis of ^{13}C-labelled 3-oxobutyl acetate, which provides an alternative efficient overall synthesis of racemic mevalonic acid. This procedure is a convenient way of producing multiply labelled mevalonic acids.*

Prior to incorporation studies, it was necessary to optimise both incorporation levels and the yield of fusicoccin formed, during fermentations to which mevalonic acid was added at the necessary high dosage levels to achieve sufficient incorporation to be detected spectroscopically. By using unlabelled mevalonic acid containing a small amount of [2-^{14}C]mevalonic acid at a dosage of 50 mg per 100 ml of culture, it was shown that the highest specific activity in the isolated fusicoccin, which coincided with the highest mass yield, occurred when the precursor was fed in one portion towards the end of the growth phase (after *ca.* 48 h) and the fermentation harvested after 7 days (see Figure). A mixture



Results of feeding [2-^{14}C]mevalonic acid (50 mg per 100 ml of culture) on the day indicated, with harvesting after 7 days

of [3-^{13}C]mevalonic acid (393 mg) and [2-^{14}C]mevalonic acid (5.54 × 10^6 disint. min^{-1}) was fed to eight 100 ml cultures of *F. amygdali* after 48 h of growth. The cultures were harvested after 7 days and the various acetylated fusicoccins in the culture extract were hydrolysed with base. The fusicoccin D (Ic) formed was separated from fusicoccins H (IIa) [5] and J (Id) [5b,6] by preparative layer chromatography. Acetylation of (Ic) with acetic anhydride in pyridine afforded ^{13}C-enriched fusicoccin triacetate (Ib) (90 mg), which was crystallised to a constant specific activity of 338 disint min^{-1} mg^{-1}. The ^{13}C n.m.r. spectrum of ^{13}C-enriched (Ib) was recorded under instrument conditions identical with those used for the natural abundance spectrum, which, with the exception of the quaternary olefinic carbon signals (C-2, -10, and -14) and the sugar resonances (C-2′ to -5′) had

TABLE 1

^{13}C Enrichment of fusicoccin triacetate (Ib)

Assignment	Chemical shift [a]	Enrichment factor [b]
C-3	48.0	3.7
C-7	40.9	3.8
C-11	55.1	2.5
C-15	32.8	4.1
C-24	75.6	3.6
Remaining signals		1.0 ± 0.2

[a] In p.p.m. to low field Me_4Si.
[b] Peak integral.

$$\left\{\frac{\text{enriched sample normalised w.r.t. C-21}}{\text{natural abundance sample normalised w.r.t. C-21}}\right\}$$

previously been completely assigned.[1] The signal intensities in both spectra were normalised with respect to C-21, which showed, as expected, no signal enhancement in the spectrum of the biosynthetic sample. The results are collected in Table 1.

* An alternative method for the formation of [3-^{13}C]mevalonic acid has recently been reported.[15]

[21] L. Canonica, L. Gaudenzi, G. Jommi, and U. Valcavi, *Gazzetta*, 1961, **91**, 1400.

[22] D. H. R. Barton, P. D. Magnus, G. Smith, G. Streckert, and D. Zurr, *J.C.S. Perkin I*, 1972, 542.

[23] J. W. Cornforth, R. H. Cornforth, A. Pelter, M. G. Horning, and G. Popjak, *Tetrahedron*, 1959, **5**, 311.

[24] R. A. Ellison and P. K. Bhatnagar, *Synthesis*, 1974, 719.

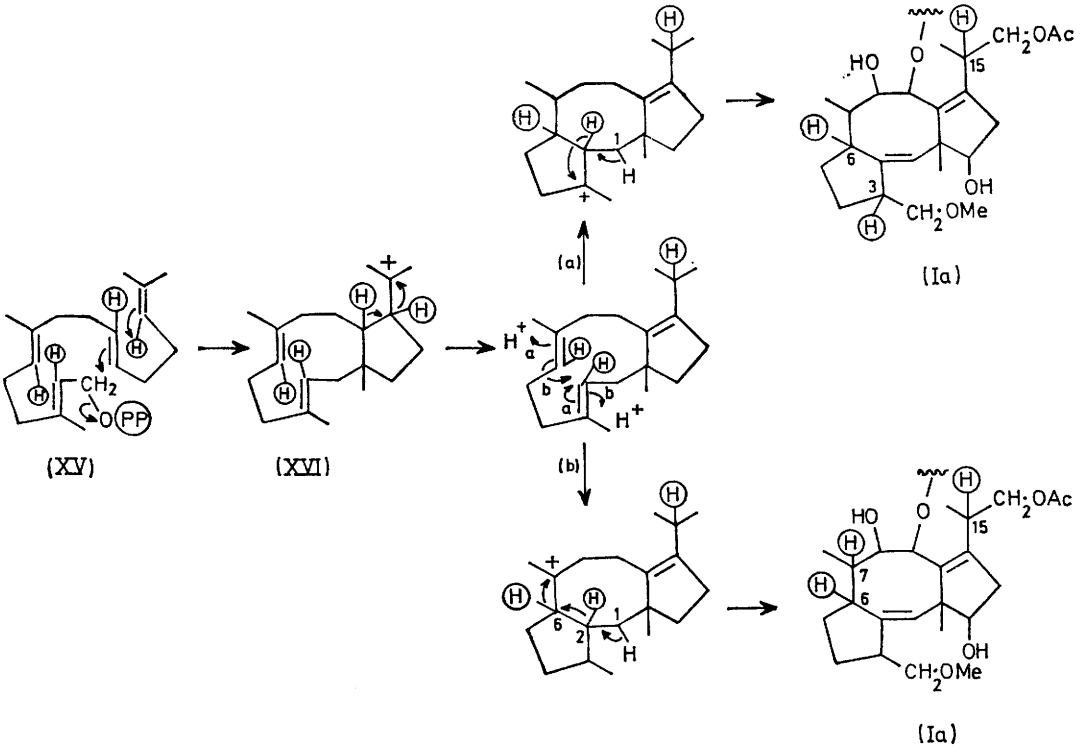
In an alternative assignment, Radics *et al.*[25] have used ^{13}C relaxation time measurements and selectively induced nuclear Overhauser enhancements to identify the signals of C-2 (144.1), C-10 (139.1), and C-14 (140.1 p.p.m.). There are, however, points of disagreement with our work, and these should be examined.

Lines corresponding to C-4, C-5, and C-13 (triplets in off-resonance decoupled spectra) were identified by us, using selective proton decoupling and lanthanide-induced shifts (C-13) and from the biosynthetic labelling pattern (C-4 and -5). Since C-5 may only be labelled by [1-^{13}C]acetate and C-4 by [2-^{13}C]acetate, we believe our assignment (C-13 34.9, C-4 28.5, C-5 36.3 p.p.m.) to be correct; Radics *et al.* reverse the assignment of C-5 and C-13. Radics *et al.* also reverse our assignment of C-8 and C-9, although we are in agreement over these peaks in the spectra of aglycone derivatives. Our assignment agrees with the biosynthetic results however (C-8 labelled by [2-^{13}C]acetate, C-9 by [1-^{13}C]acetate), and the alternative does not.

The C-18 and C-20 signals present a more difficult problem. Again the respective assignments are reversed. Radics *et al.* resolve the problem in terms of the observed chemical shifts, and indeed we do state [1] that these are more in accord with their conclusion. However, in a molecule of unknown and probably complex conformation it may be safer to rely on the fact that the line at 15.8 p.p.m. is a much sharper quartet in off-resonance decoupled spectra than that at 24.9 p.p.m., and hence to assign it to C-18. The biosynthetic arguments in this and the previous paper [1] are not affected by this dichotomy.

We should point out here a typographical error in Table 4 of reference 1. In column 7, the + sign which is fourth from the top of the list should be deleted and placed on the same line in column 8.

With the exception of C-11, the enrichment factors of the five enriched positions, C-3, C-7, C-11, C-15, and C-24, are in good agreement with the anticipated value of 3.8 calculated from the ^{14}C data. The apparently low enrichment at C-11 is probably an artefact arising from the difficulty of obtaining reproducible signal intensities for quaternary carbons in different samples. Carbon atom 11 will probably have the longest relaxation time of all the enriched carbons (although C-24 is quaternary, it has more nearest neighbour hydrogen atoms for relaxation by the dipolar mechanism) and hence its intensity will be most susceptible to the influence of dissolved paramagnetic impurities (metal ions or oxygen) and variation in the pulse width from one experiment to the other. Subsequent experiments (to be reported elsewhere), in which the system was saturated with oxygen as a relaxation aid, support this view.



SCHEME 4

The incorporation of [3-^{13}C]mevalonic acid into C-3, C-7, C-11, C-15, and C-24 of (Ia) is in full agreement with the conclusion drawn from the labelling of (Ia) by

[25] L. Radics, M. Kajtar-Peredy, C. G. Casinovi, G. Grandolini, and C. Rossi, *Org. Magnetic Resonance*, 1975, **7**, 137.

[1-^{13}C]- and [2-^{13}C]-acetate [1] and provides further evidence for a mechanism of cyclisation of geranylgeranyl pyrophosphate (XV), similar to that of geranylfarnesyl pyrophosphate (V) cyclisation in ophiobolin F biosynthesis,[26] in which the integrity of the carbon–carbon bonds is retained. However, the unsaturation and oxygen pattern of fusicoccin suggested that the further cyclisation of the bicyclic carbocation (XVI) (or its biological equivalent), analogous to (VI), might proceed by an alternative route. To test this notion (4*R*)-[2-^{14}C, 4-$^{3}H_1$]mevalonic acid was fed to the fungus. If the ophiobolin type cyclisation was operative, only two out of a possible five tritium atoms would be expected to be retained in (Ia), located at C-16 and in the isolated isoprenoid residue. The results of two independent feeding experiments are collected in Table 2.

TABLE 2

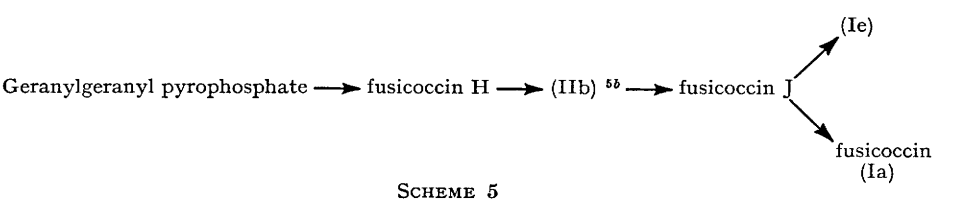
Incorporation of (3*RS*,4*R*)-[4-^{3}H, 2-^{14}C]Mevalonic acid (MVA) into fusicoccins

	^{3}H : ^{14}C Ratio 4*R*-[4-^{3}H, 2-^{14}C]MVA		Normalised ^{3}H : ^{14}C atom ratio				
Expt.			Fusicoccin (Ia)	Fusicoccin triacetate (Ib)	Fusicoccin H (IIa)	Fusicoccin J (Id)	Incorporation * (%)
1	12.5 : 1	5 : 5	3.76 : 5		3.30 : 4	3.76 : 5	0.036 †
2	6.78 : 1	5 : 5		3.92 : 5			8.24

* Calculated from ^{14}C data and including allowance for utilisation of 3*R* enantiomer of MVA only. † Calculated for fusicoccin.

Clearly only one tritium atom is lost during the cyclisation of geranylgeranyl pyrophosphate and ensuing modifications. This result can be explained by the further cyclisation of the bicyclic carbocation ion intermediate (XVI) in one of two ways [Scheme 4 (a) and (b)], which differ in the direction of carbon–carbon bond formation between C-2 and C-6. In each case the resulting carbocation (or its biological equivalent) would be quenched by a process involving the initial loss of a proton from C-1. In route (b) this would be followed by either two 1,2-hydride shifts or a 1,3-hydride shift, whereas route (a) requires only one 1,2-hydride shift.

The formation of the recently characterised cometabolite (Ie) could arise by the enzymic hydroxylation of fusicoccin J (Id) at C-3, representing a deviation from the main pathway to fusicoccin * as depicted in Scheme 5.



SCHEME 5

With regard to di- and sester-terpenes with the same carbocation as the fusicoccin aglycone, it is tempting to speculate that those with a double bond at position 7 and unsaturation or a hydroxy-group at position 3 arise through the ophiobolin cyclisation route, whereas those with unsaturation at C-1 and C-10(14) could evolve through the alternative cyclisation mode.

* Both fusicoccins H and J have previously been shown to be incorporated into fusicoccin,[5,6] but details of feeding experiments with (Ie) and (IIb) [5b] are not available.

EXPERIMENTAL

Carbon-13 n.m.r. spectra were recorded at 25.16 MHz on a Varian XL-100-12–Varian Data Machine 16 K 620-L spectrometer system (8 K sampling points after Fourier transformation). Samples were dissolved in deuteriochloroform (2 ml) in a 10 mm n.m.r. tube, the solvent deuterium being used to provide a field frequency lock. ^{1}H N.m.r. spectra were recorded for solutions in deuteriochloroform with a Varian T-60 spectrometer and i.r. spectra with a Unicam SP 800 spectrometer. Preparative t.l.c. was carried out on silica gel GF_{254} (1 mm thick) which had been activated at 100 °C for 1.5 h.

[1-^{13}C]*Acetyl Chloride*.—Sodium [1-^{13}C]acetate (92 atom %, 9.33 g; dried at 210 °C and 0.1mmHg for 48 h) and redistilled phthaloyl chloride (80 ml) were heated with stirring on an oil-bath. As the bath temperature reached 160 °C, [1-^{13}C]acetyl chloride began distilling over (b.p. 50—53 °C) into a flask cooled to −70 °C. The last traces were driven over by heating with a free flame (yield 8.32 g).

N-[1-^{13}C]*Acetylimidazole*.—A mixture of imidazole (13.6 g), hexamethyldisilazane (24.6 g), and concentrated sulphuric acid (2 drops) was heated under reflux for 2 h. *N*-Trimethylsilylimidazole was obtained by fractional distillation; b.p. 87° at 10 mmHg.

[1-^{13}C]Acetyl chloride (3.00 g) was added dropwise to a solution of *N*-trimethylsilylimidazole (5.34 g) in dry benzene at 0 °C. The mixture was stirred during 45 min. The solvent was removed *in vacuo* at 35 °C and the residual, vacuum dried *N*-[1-^{13}C]acetylimidazole (6.57 g) was used in the next reaction without further purification; τ 7.29 [3 H, d, J(^{13}C–^{1}H) 7 Hz].

Ethyl [3-^{13}C]*Acetoacetate*.—A mixture of ethyl hydrogen malonate (8.09 g), magnesium ethoxide (7.01 g; dried at 100 °C and 0.1 mmHg for 24 h), and dry tetrahydrofuran (125 ml) was heated, with stirring, on an oil-bath (70—73 °C) for 1 h. The solvent was removed *in vacuo* at 50—60 °C. The residue was stirred with dry benzene (30 ml) and the

[26] S. Nozoe and M. Morisaki, *Chem. Comm.*, 1969, 1319.

solvent similarly removed. This procedure was repeated once more, yielding a dry powder. Dry tetrahydrofuran (140 ml) was added to the powder and the mixture stirred at room temperature while a solution of *N*-[1-^{13}C]acetylimidazole (6.57 g) in dry tetrahydrofuran (80 ml) was added dropwise. The mixture was stirred at room temperature during 2½ h, then the solvent was removed *in vacuo* at 30—40 °C. The residue was carefully treated with concentrated hydrochloric acid (11.5 ml) in water (2 ml) until the pH was *ca.* 6. The acidified solution was saturated with salt and extracted with ether (4 × 60 ml). Evaporation of the combined, dried extracts yielded crude ethyl [3-^{13}C]-acetoacetate (5.34 g). A second experiment with ethyl hydrogen malonate (6.099 g), magnesium ethoxide (5.22 g), and *N*-[1-^{13}C]acetylimidazole (4.95 g) afforded more ethyl [3-^{13}C]acetoacetate (4.11 g). The crude products were combined and distilled to give pure ethyl [3-^{13}C]acetoacetate (8.85 g, 63%), b.p. 77—78.5° at 19 mm Hg, τ 8.79 (3 H, t, *J* 7 Hz), 8.07 [d, *J* (^{13}C–^{1}H) 6 Hz, enol Me (7%)], 7.79 [3H, d, *J*(^{13}C–^{1}H) 6 Hz, keto Me (93%)], 6.52 [d, *J*(^{13}C–^{1}H) 6 Hz, keto 2-H (93%)], 5.85 (2 H, q, *J* 7 Hz), and 4.93 [d, *J*(^{13}C–^{1}H) 6 Hz, enol 2-H (7%)].

Ethyl [3-^{13}C]*Acetoacetate Ethylene Acetal.*—A mixture of ethyl [3-^{13}C]acetoacetate (5.83 g), ethylene glycol (2.98 g), and concentrated sulphuric acid (33 mg) in dry benzene (30 ml) was heated under reflux, with a Dean–Stark column, for 2½ h. Removal of solvent *in vacuo* gave the acetal (7.76 g), τ 8.82 (3 H, t, *J* 6 Hz), 8.57 [3 H, d, *J*(^{13}C–^{1}H) 5 Hz, 4-Me], 7.42 [2 H, d, *J*(^{13}C–^{1}H) 5 Hz, 2-H], 6.12 [4 H, d, *J* (^{13}C–^{1}H) 3 Hz, acetal H], and 5.95 (2H, q, *J* 6 Hz).

4-*Hydroxy*[2-^{13}C]*butan-2-one Ethylene Acetal.*—Ethyl [3-^{13}C]acetoacetate ethylene acetal (7.76 g) was added dropwise to stirred lithium aluminium hydride (2.29 g) in dry ether (60 ml) at room temperature. The mixture was heated under reflux for 2½ h. Water (10 ml) was added with stirring and the sludge diluted with ether (200 ml) and stirred until the ether was non-turbid. The mixture was filtered through sand. Evaporation of the dried filtrate gave the [2-^{13}C]alcohol (5.31 g), τ 8.70 [3 H, d, *J*(^{13}C–^{1}H) 5 Hz], 8.10 (2 H, m), 6.14 (3 H, m, OH, and 1-H), and 6.04 [4 H, d, *J*(^{13}C–^{1}H) 3 Hz].

3-*Oxo*[3-^{13}C]*butyl Acetate Ethylene Acetal.*—A mixture of 4-hydroxy[2-^{13}C]butan-2-one ethylene acetal (4.98 g), redistilled acetic anhydride (4.5 ml), and redistilled dry pyridine (7.0 ml) was left at room temperature for 24 h. The mixture was evaporated with methanol (5 ml) under reduced pressure and the pyridine and acetic acid were carefully removed *in vacuo* at 30—40 °C and 0.5 mmHg to give the [3-^{13}C]acetate (6.53 g), τ 8.73 [3 H, d, *J*(^{13}C–^{1}H) 5 Hz], 8.02 (3 H, s), 8.00 (2 H, m), 6.12 [4 H, d, *J*(^{13}C–^{1}H) 3 Hz], and 5.95 (2 H, m); *m/e* 160 (M^+ — Me), 116, 110, 88, 44, and 43.

3-*Oxo*[3-^{13}C]*butyl Acetate* (XI).—A mixture of the foregoing acetal (3.33 g), trityl tetrafluoroborate [27] (9.72 g), anhydrous sodium carbonate, and dry methylene chloride (65 ml) was stirred at room temperature for 1 h. Water (2.3 ml) was added to the orange solution and the mixture stirred for 30 min. Methylene chloride (200 ml) was added and the organic layer was washed with cold saturated brine (2 ml). Evaporation of the dried methylene chloride extract yielded a semi-solid (10.46 g). A second experiment with labelled acetal (2.25 g), trityl tetrafluoroborate (5.83 g), anhydrous sodium carbonate (0.99 g), and dry methylene chloride (60 ml) afforded crude product (6.28 g). The crude products from the two experiments were combined and distilled to give 3-oxo[3-^{13}C]butyl acetate (2.60 g), b.p. 82—92° at 19 mmHg, τ 7.99 (3 H, s), 7.82 [3 H, d, *J*(^{13}C–^{1}H) 5 Hz], 7.20 (2 H, m), and 5.72 (2 H, m).

[3-^{13}C]*Mevalonolactone.*—A solution of the foregoing acetate (2.42 g) in dry ether (5 ml) was cooled to −30 °C, and redistilled boron trifluoride–ether (0.06 ml) was added. Keten (slightly over 1 equiv.) was bubbled through the ethereal solution from a two-way stream splitter connected with the keten generator. The other lead from the two-way valve led into an equivalent amount of standard N-sodium hydroxide containing phenolphthalein, and the leads were used alternately for 2 min each. The time required to neutralise the standard alkali was taken as the time for the reaction. The mixture was left at −25 °C for 1½ h and then added with the aid of more methanol to methanolic N-potassium hydroxide (57 ml) precooled to −15 °C. The pale yellow solution was kept overnight at room temperature, then neutralised with methanolic hydrogen chloride until slightly basic (Phenol Red). The solvent was removed and the residue, after washing with dry ether (3 × 8 ml) to remove neutral material, was treated with methanol (15 ml) and then with sufficient methanolic hydrogen chloride to bring the total acid added to 56.3 mequiv. (0.7 ml N-potassium hydroxide consumed by 0.056 ml boron trifluoride–ether). Potassium chloride was removed by filtration and the residue extracted with chloroform. Evaporation of the extract gave a yellow oil (2.30 g) which was dissolved in ether and treated with ethereal diazomethane until no effervescence was observed. Evaporation of the dried solution afforded a residue which was kept under vacuum at 35—40 °C and 0.05 mmHg to remove methyl 3-hydroxyisovalerate and other volatile impurities. The oily residue (1.25 g) in chloroform was chromatographed on silica gel MFC (190 × 20 mm). The column was eluted with chloroform and 50 ml fractions were collected. Fractions 1—3 contained small amounts of impure [3-^{13}C] mevalonolactone. Fractions 4—7 (0.51 g) and 8—19 (0.40 g) yielded pure [3-^{13}C]mevalonolactone, τ ($CDCl_3$) 8.62 [3 H, d, *J*(^{13}C–^{1}H) 5 Hz, Me], 8.05 (2 H, m, 4-H), 7.40 (2 H, m, 2-H), 6.10 (1 H, s, OH), and 5.50 (2 H, m, 5-H); δ_C (p.p.m. from Me_4Si) 29.6 (d, *J* 40 Hz, 1%, CH_3), 35.8 (d, *J* 37.3 Hz, 1%, C-4), 44.6 (d, *J* 36.5 Hz, 1%, C-2), 66.2 (d, *J* 2.1 Hz, 1%, C-5), 68.1 (s, 90%, C-3), and 171.1 (s, 1%, C-1); *m/e* 131 (M^+), 116 (M^+ — Me), 113 ($M^+ - H_2O$), 104 (M^+ — 27), 103 (M^+ — 28), 72 (M^+ — 59), 59, and 44. The i.r. spectrum and g.l.c. retention time of the [3-^{13}C]mevalonolactone were identical with those of an authentic sample.

5-*Acetoxy*-3-*methylpentan*-3-*olide* (XIII).—3-Oxobutyl acetate (1.94 g) in dry ether (5 ml) was cooled in a bath at −30 °C, and redistilled boron trifluoride–ether (0.045 ml) was added. Keten (slightly over 1 equiv.) was passed through the mixture, which was then left at −25 °C for 2 h. The yellowish crystalline solid (1.57 g) was filtered off. Attempts to recrystallise a portion of this from ether–light petroleum (b.p. 40—60 °C) led to its rapid polymerisation. The remaining material was washed with cold ether–light petroleum to yield the *lactone* (XIII) as prisms, m.p. 38—41°, $\nu_{max.}$ ($CHCl_3$) 1 822 and 1 742 cm^{-1}, τ 8.35 (3 H, s), 7.93 (3 H, s), 7.85 (2 H, m), 6.71 (2 H), and 5.73 (2 H, t, *J* 6 Hz) (Found: C, 55.8; H, 7.0. $C_8H_{12}O_4$ requires C, 55.8; H, 7.0%).

[27] H. J. Dauben, jun., L. R. Honnen, and K. M. Harmon, *J. Org. Chem.*, 1960, **25**, 1442.

General Methods for Feeding Experiments.—A high-yielding strain of *Fussicoccum amygdali* Del., derived by u.v.-induced mutation was inoculated into 500 ml conical flasks containing 100 ml of sterile medium of the following composition: potato infusion (30%), glucose (3%), tap water to final volume. The cultures were shaken for 4 days at 24 °C. The culture filtrate was decanted off and the mycelium homogenised in a sterile Waring blender. Samples (10 ml) of the resulting homogenate were then used to inoculate flasks containing 100 ml of sterile medium of the following composition: glucose (3%), sodium nitrate (0.33%), potassium dihydrogen phosphate (2%), potassium chloride (0.05%), magnesium sulphate heptahydrate (0.05%), ferrous sulphate heptahydrate (0.001%), soya bean meal (0.2%), tap water to final volume. The secondary cultures were incubated at 24 °C for 7 days. Precursors were added at the appropriate times. After harvesting, the mycelium was separated from the culture filtrate by filtration through muslin and the filtrate extracted by the appropriate means (see below).

Incorporation of (4R)-[4-^{3}H,2-^{14}C]*Mevalonolactone.*—[2-^{14}C]Mevalonolactone (2 μCi; 135 μCi mg^{-1}) was mixed with (4*R*)-[4-^{3}H]mevalonolactone in water (2 ml) (1.9 mCi mg^{-1}) to give a ^{3}H : ^{14}C ratio of 12.5 : 1. This was fed to a 1 l culture of *F. amygdali* in a 5 l conical flask after 24 h growth. The culture was harvested after 4 days. Fusicoccin (90 mg) and fusicoccin H (90 mg) were added to the culture filtrate, which was successively extracted with light petroleum, chloroform, and butan-1-ol. The chloroform extract was rich in the various fusicoccins; the butanol extract primarily contained fusicoccins H and D. Fusicoccin and fusicoccin J (after dilution of the extract with 17.2 mg of inactive material) were isolated from the chloroform extract by p.l.c. [chloroform–methanol–water (100 : 8 : 1 v/v)] Fusicoccin H was similarly separated from the butanol extract by p.l.c. [chloroform–methanol–water (100 : 20 : 1 v/v)]. All three fusicoccins were crystallised to constant specific activity.

In a second experiment (4*R*)-[4-^{3}H, 2-^{14}C]mevalonolactone (3.08 μCi ^{14}C; ^{3}H : ^{14}C 6.01 : 1) was fed to four 100 ml cultures of the fungus which were harvested after 7 days. The culture filtrate was extracted with isobutyl methyl ketone. The evaporated and dried extract was treated with acetic anhydride (1 ml) in pyridine (2 ml) overnight at room temperature. After work-up the product was chromatographed on a column of silica gel MFC (30 g) prepared in benzene. The column was eluted with benzene (200 ml), benzene–chloroform (1 : 1 v/v; 200 ml), and chloroform (300 ml). Fusicoccin triacetate was eluted between 300 and 500 ml of eluate. This fraction crystallised from chloroform–light petroleum (b.p. 60—80 °C) to give material (83.0 mg) which was recrystallised to constant specific activity (3 further crystallisations). The results are collected in Table 2.

Trial Incorporation of [2-^{14}C]*Mevalonic Acid into Fusicoccin.*—Mevalonolactone (50 mg per 100 ml of culture), to which had been added a small quantity of [2-^{14}C]mevalonolactone (2 μCi per 100 ml of culture) was added to growing cultures of the fungus at various time intervals. The fermentations were harvested after 4 or 7 days. The fusicoccin extract was treated with base. Fusicoccin D was isolated (*ca.* 60 mg for five 100 ml cultures) by p.l.c. and crystallised to constant specific activity. The results are collected in Table 3.

TABLE 3

Feeding day	Harvesting day	Fusicoccin D (disint. min^{-1} mg^{-1})	Incorporation (%)
1	4	8.86×10^{2}	0.22
1	7	1.37×10^{3}	0.34
2	4	1.34×10^{3}	0.33
2	7	1.90×10^{3}	0.48
3	7	1.88×10^{3}	0.46
4	7	1.12×10^{3}	0.28

Incorporation of [3-^{13}C]*Mevalonolactone.*—A solution of [3-^{13}C]mevalonolactone (393 mg) containing [2-^{14}C]mevalonolactone (5.54×10^{6} disint. min^{-1}) in water (8 ml) was added to eight 100 ml cultures of the fungus which had been growing for 48 h. After 7 days the cultures were harvested and worked up by the procedure outlined for the second feeding of (4*R*)-[4-^{3}H,2-^{14}C]mevalonolactone (see above). The ^{13}C-enriched fusicoccin triacetate (90 mg, 3.38×10^{2} disint. min^{-1} mg^{-1}) obtained was examined by ^{13}C n.m.r. spectroscopy. The results are in Table 1.

We thank Sir Derek Barton and Sir Ernst Chain for their interest in this work, the S.R.C. for financial support towards the purchase of the Varian XL-100-12 spectrometer and a maintenance award (to R.B.J.), and the International Atomic Energy Agency for a Senior Fellowship (to A. B.).

[6/624 *Received*, 1*st April*, 1976]