Fusicoccin. Part V.[1] The Biosynthesis of Fusicoccin from [1-^{13}C]- and [2-^{13}C]-Acetate

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The positions of labelled atoms in fusicoccin biosynthesised from [1-^{13}C]- and [2-^{13}C]-acetate have been determined by ^{13}C n.m.r. spectroscopy. The results are consistent with fusicoccin being formed by direct cyclisation of a precursor such as geranylgeraniol pyrophosphate.

We have previously shown that the minor metabolite fusicoccin H (I) can act as a biosynthetic precursor of fusicoccin (IIa), the major wilting toxin of the plant pathogen fungus *Fusicoccum amydali Del.*, and have argued that this provides evidence that fusicoccin (IIa) is a diterpene, not a degraded sesterterpene.[2] We now present evidence that fusicoccin is formed by cyclisation of a terpenoid precursor such as a geranylgeraniol pyrophosphate.

The terpenoid nature of the fusicoccin aglycone and side chain was confirmed in our early work by feeding [2-^{14}C]mevalonic acid.[3,4] A considerable amount of new chemistry would have to be undertaken to locate and verify the expected positions of the labelled carbon atoms. A simpler approach is to use the non-radioactive ^{13}C isotope and ^{13}C n.m.r. to locate the positions enriched in ^{13}C.

The success of this type of experiment depends upon obtaining incorporations high enough to be detected spectroscopically. This is the major limitation of such a study, as the massive amounts of precursors that must be added to give high incorporations can perturb or interrupt the delicate biological system which may have been carefully set up to give maximum yield of the metabolite under study. In the case of rifamycin biosynthesis [5] adequate incorporations could only be obtained by adding small amounts of ^{13}C precursor at regular intervals. Fungal metabolites are generally produced more slowly, taking days or weeks to reach maximum yields and so are quite amenable to this type of experiment.

Secondary metabolites are usually produced by microorganisms in the late exponential phase of growth, *i.e.* after most of the growth of the organism has occurred,[6] so we used an alternative procedure and added our precursors just as fusicoccin production was starting, and hoped to avoid serious interference with the growth. The total yield of fusicoccins in the various acetylated forms [3] was reduced 3—4 fold, but was still adequate for our purpose. For experimental convenience we isolated fusicoccin (IIa) as its triacetate (IIb), which crystallised directly from the crude reaction product. The results of the spectral

[1] Part IV, K. D. Barrow, D. H. R. Barton, Sir Ernst Chain, D. Bageenda-Kasujja, and G. Mellows, *J.C.S. Perkin I*, 1975, 877.

[2] K. D. Barrow, D. H. R. Barton, Sir Ernst Chain, U. F. W. Ohnsorge, and R. P. Sharma, *J.C.S. Perkin I*, 1973, 1590.

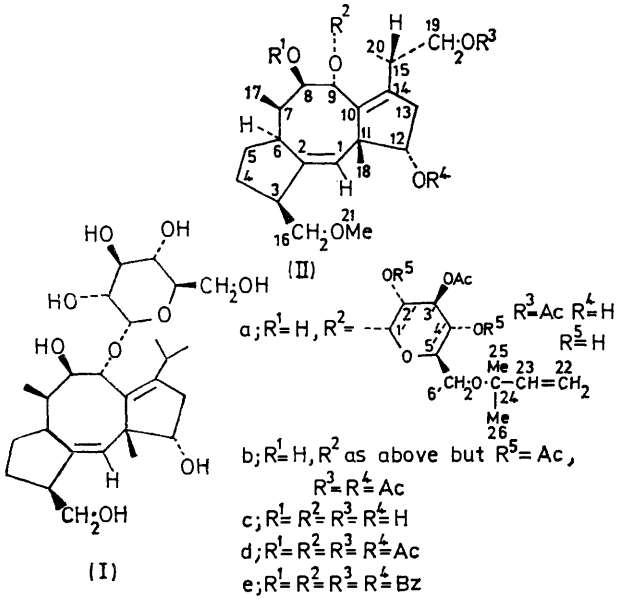
[3] K. D. Barrow, D. H. R. Barton, Sir Ernst Chain, C. Conlay, T. C. Smale, R. Thomas, and E. S. Waight, *J. Chem. Soc.* (*C*), 1971, 1259.

[4] K. D. Barrow, D. H. R. Barton, Sir Ernst Chain, U. F. W. Ohnsorge, and R. Thomas, *J. Chem. Soc.*, (*C*) 1971, 1265.

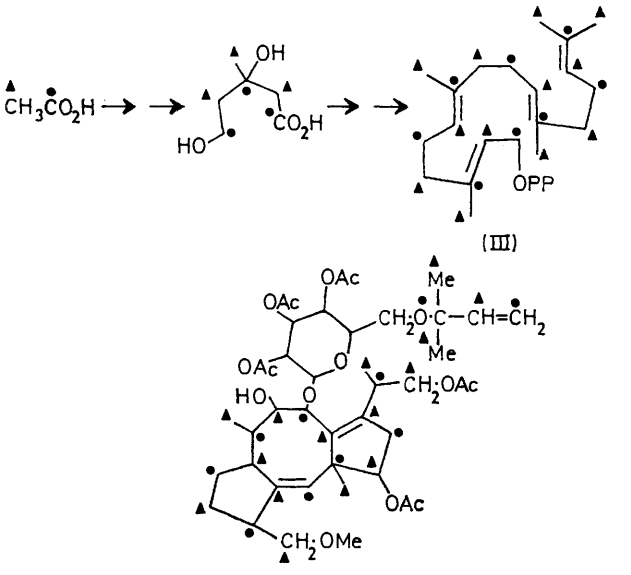
[5] R. J. White, E. Martinelli, G. G. Gallo, G. Lancini, and P. J. Beynon, *Nature*, 1973, **243**, 273.

[6] J. D. Bu'Lock, D. Shepherd, and D. J. Winstanley, *Canad. J. Microbiol.*, 1967, **15**, 279.

assignments and the feeding experiments with [1-^{13}C]- and [2-^{13}C]-acetate are shown in Table 4. This



clearly shows ten signals enhanced 5-fold after feeding [1-^{13}C]acetate, and fifteen signals enhanced 3-fold after feeding [2-^{13}C]acetate. This is consistent with fusicoccin being formed by cyclisation of a precursor such as geranylgeraniol pyrophosphate (III) (Scheme 1). This process bears resemblance to that producing the sesterterpene ophiobolin F (VI) [7] from geranylfarnesyl pyrophosphate (IV) (Scheme 2), but the unsaturation and oxygen

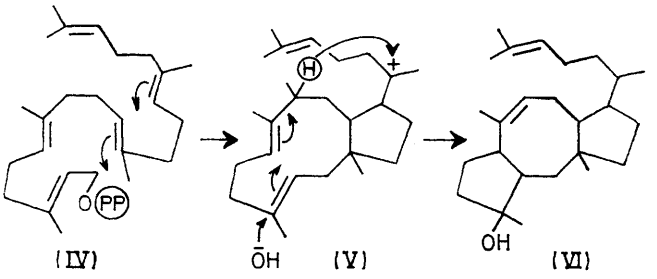


SCHEME 1

pattern of fusicoccin can also be explained by alternative routes from a bicyclic carbocation analogous to (V).

[7] L. Canonica, A. Fiecchi, M. Galli Kienle, B. M. Ranzi, A. Scala, T. Salvatori, and E. Pella, *Tetrahedron Letters*, 1967, 3371.

The assignment of the ^{13}C n.m.r. spectrum of the triacetate (IIb), achieved by using the derivatives (IIc—e) for comparisons, is detailed below. We were unable to assign the quaternary olefinic carbon signals [C-2, C-10, and C-14, δ_C 144.1, 140.1, and 139.1 (p.p.m. to low field of Me_4Si)], but this does not affect the biosynthetic result as all three are labelled by [2-^{13}C]acetate. The situation is complicated because these are all bridgehead positions in fused rings, and the chemical shifts appear to be very susceptible to steric distortions. No attempt was made to assign the sugar carbon atoms C-2′—5′ as these are not involved in the terpenoid biosynthesis. The C-4 and C-5 signals (δ_C 28.5 and 36.3) could not be assigned individually by spectral methods or correlation. However, one of C-4 and C-5 is labelled by [1-^{13}C]acetate and the other by [2-^{13}C]acetate, so if we accept that fusicoccin is a normally cyclised terpene (*i.e.* without backbone rearrangements), C-5 resonates at δ_C 36.3 and C-4 at 28.5. These are the only two of the 25 terpenoid carbon atoms in fusicoccin for which biosynthetic assumptions were



SCHEME 2

made to enable spectral assignments. Carbon atoms 25 and 26 could not be assigned, but as again they are both labelled by [2-^{13}C]acetate and not by [1-^{13}C]acetate, the assignment does not affect the biosynthetic argument.

Fusicoccin Aglycone Tetrabenzoate (IIe).—The main features of the ^{1}H n.m.r. spectrum of (IIe) have been assigned [4] and the ^{13}C n.m.r. spectrum may be related to this by the incremented heteronuclear decoupling technique.[8] A coherent secondary irradiating field at ^{1}H resonance frequencies was moved, in increments of 0.50 p.p.m., from 0.50 to 6.50 p.p.m. to high frequency (low field) of the Me_4Si resonance while successive ^{13}C spectra were recorded. A plot of the residual $^1J_{CH}$ upon a given ^{13}C resonance *vs.* the ^{1}H irradiation frequency passes through a minimum corresponding to the chemical shift of the directly bonded proton; the carbon may then be assigned.

The ^{1}H noise-decoupled ^{13}C n.m.r. spectrum shows the 21 expected resonances from the ' backbone ' plus partially resolved lines due to the benzoate functions (carbonyl resonances appear at δ_C 165.2, 165.9, 166.1, and 166.2 and the ring carbon atoms absorb in the range δ_C 128.0—133.1). High-power single-frequency off-resonance (proton) decoupling (s.f.o.r.d.) enabled the number of protons bonded to each carbon atom to be deduced, and

[8] B. Birdsall, N. J. M. Birdsall, and J. Feeney, *J.C.S. Chem. Comm.*, 1972, 316.

in addition to the benzoate resonances the spectra show the following details.

Olefinic resonance region. This showed four signals, three of which arise from the quaternary C-2, C-10, and C,14 (δ_C 138.0, 143.6, and 144.2) and one from C-1 (127.4, doublet in the s.f.o.r.d. spectrum; optimum decoupling occurs at a proton frequency corresponding to δ 4—5).

'Carbon bonded to oxygen' region. There are six resonances in this region, and that due to C-21 is easily recognised (δ_C 58.7, quartet); optimum decoupling at δ 3.35 [δ(H-21) is 3.32]. Two lines which are triplets in s.f.o.r.d. occur at δ_C 67.4 and 76.5. The latter is assigned to C-16 because of its similar chemical shift in the spectra of compounds (IIc and d); this is supported by the incremented decoupling experiment, although poorly defined line-shapes necessitated a visual estimation of the optimum decoupling frequency in each case {δ(C-16) 76.5, optimum decoupling at δ *ca.* 3.4 [δ(H-16) 2.9—3.5 (multiplet)]; δ(C-19) 67.4, optimum decoupling at δ *ca.* 4.0 [δ(H-19) not assigned]}.

The three remaining signals appear as residual doublets on s.f.o.r.d. The lowest field of these (δ_C 80.7) is relatively sharply defined, and since optimum decoupling occurred at δ 5.2 [δ(H-12) is 5.3] is assigned to C-12. The resonances due to C-8 and C-9 appear as characteristic 'doublets of doublets' from which very poor plots of $^1J_{CH}$ (residual) *vs.* proton decoupling frequency were obtained. This type of band-shape has been reported previously for off-resonance decoupling experiments in which the carbon nucleus is the X part of an ABX spin system,[9] and is a consequence of the strong proton coupling between A and B. Visual estimation of the optimum decoupling frequency suggests that the resonance due to C-8 occurs at δ_C 77.2, and that due to C-9 at 66.9.

'Unsubstituted aliphatic' region. Many of the signals here show considerable distortion in s.f.o.r.d. spectra, because of the second-order nature of the proton system to which they are coupled. We have noted three degrees of line definition as follows. (*a*) When the ^{13}C nucleus is coupled to proton(s) which are part of a tightly coupled spin system a great distortion (unresolved broadening or fine structure) is observed. (*b*) When the ^{13}C nucleus is coupled to proton(s) which form part of a loosely coupled spin system, line-broadening occurs, but the multiplet is well defined. (*c*) When the ^{13}C nucleus is coupled to proton(s) which are not further coupled to other protons, the narrowest lines occur.

These line-shapes proved of great diagnostic value, both for estimating the nature of the inter-proton spin system to which the carbon nucleus is coupled (as with C-8 and C-9 above) and as an aid to relating spectra of different molecules (*e.g.* the C-12 signal may immediately be distinguished from those of C-8 and C-9 in all spectra by the better definition of its residual doublet).

The difference between situations (*b*) and (*c*) is subtle, and best seen by comparison within a given spectrum (see Figure 1). By way of confirmation, we have demonstrated such an effect in the s.f.o.r.d. spectrum of ethyl acetate; here the residual quartet due to $CH_3{\cdot}CO$ is appreciably narrower than that due to $CH_3{\cdot}CH_2$.

Three residual quartets are observed, corresponding to the methyl carbon atoms C-17, C-18, and C-20. The signal at δ_C 11.4 showed optimum decoupling at δ 1.15, and since δ(H-17) is 1.20 and δ(H-20) 1.11, may be assigned to either C-17 or C-20. The lines at δ_C 15.3 and 24.9 showed optimum decoupling at δ 1.40—1.43 and 1.30—1.35, respectively. Previous assignment of the ^1H spectrum gave the chemical shift of H-18 as 1.38, that of H-17 as 1.20, and that of H-20 as 1.11. This is inconsistent with the present results and the ^1H spectrum was re-examined.

In the ^1H spectrum, H-7 absorbs at δ_H 2.22; irradiation here with a (homonuclear) decoupling field collapses the highest field doublet (δ 1.12, $^3J_{HH'}$ 7 Hz), and this therefore corresponds to the C-17 protons. The H-15 signal was not identified in the spectrum, but the most probable absorption occurs at δ 4.25 and irradiation here causes a partially hidden doublet (δ_H 1.42, J_{HH} 7 Hz) to collapse to a singlet. This is therefore assigned to the C-20 protons. The C-18 protons absorb as a singlet at δ_H 1.40.

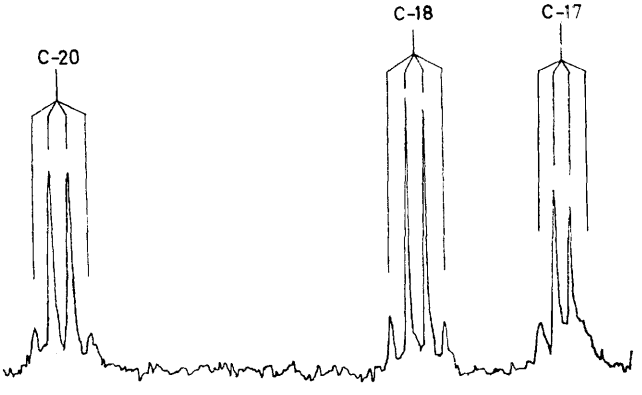


FIGURE 1 Fusicoccin aglycone tetrabenzoate ^{13}C n.m.r. spectrum, single frequency (^1H) off-resonance decoupled; high-field methyl region, to illustrate different line-shapes

When these results are related to the ^{13}C spectrum, the C-17 signal (at δ_C 11.4) may be assigned, but those of C-18 and C-20 may not because of the close proximity of their proton resonances. In the s.f.o.r.d. spectrum, however, the quartet at δ_C 15.3 p.p.m. is significantly narrower and taller than that at 24.9 (Figure 1); the former may then be assigned to C-18 and the latter to C-20. Similar line-shape differences were observed for these resonances in the s.f.o.r.d. spectra of (IIb—d) and this provides a further example of the importance of the subtle difference between cases (*b*) and (*c*) above. However the chemical shifts of C-18 and C-20 are probably more in accord with the alternative assignment.

Three residual triplets are observed, corresponding to C-4, C-5, and C-13. That at δ_C 35.2 possessed the better defined line-shape, and since optimum decoupling occurred at δ 2.70—2.75 it was assigned to C-13 [δ(H-13α,

[9] R. A. Newmark and J. R. Hill, *J. Amer. Chem. Soc.*, 1973, **95**, 4435.

H-13β) 2.83 and 2.53]. The other triplets were too distorted for a successful plot of $^1J_{CH}$ residual *vs.* decoupling frequency to be made, but a visual estimate of the optimum decoupling frequency was δ 1.5. This is consistent with their assignment to C-4 and C-5 [δ(H-4α, H-4β, H-5α, H-5β) all in the range 1—2]; the relative invariance of these signals to the substituent on C-12 (unlike the C-13 signal, which moves upfield by the expected amount) confirms this. The C-4 and C-5 signals may not be distinguished from each other at this or any later stage, and the assignment was only achieved by consideration of the pattern of labelled acetate incorporation.

Four residual doublets are observed on s.f.o.r.d., corresponding to C-3, C-6, C-7, and C-15. The ^{1}H n.m.r. assignment of H-7 and H-15 has been described above, and this enables the assignment of a signal at δ_C 32.6 to C-15 [optimum decoupling at δ 4.3; δ(H-15) 4.25], and that at 40.7 to C-7 [optimum decoupling at δ 2.2, δ(H-7) 2.22]. The H-3 and H-6 signals may be identified, but not distinguished, by their $^4J_{HH}$ (allylic) coupling to H-1, and in the ^{1}H n.m.r. spectrum they occur at δ 3.00 and 2.64; optimum decoupling of the ^{13}C doublet at δ_C 41.4 occurred at δ 3.1 and of that at 47.6 at δ 2.45. This indicates that the lower field proton is bonded to the higher field carbon atom, and *vice versa*.

Of the pair of doublets corresponding to C-3 and C-6, the higher-field doublet appears the better defined (less second-order effects). Inspection of the ^{1}H spectrum indicates that H-6 is part of a more weakly coupled homonuclear spin-system than is H-3; the δ_C 41.4 line may thus be assigned to C-6, and that at 47.6 to C-3.

The signal due to the single quaternary carbon atom, C-11, is easily identified from the s.f.o.r.d. spectrum, and appears at δ_C 56.8.

With the exception of the olefinic carbon atoms C-2, C-10, and C-14, and the differentiation between C-4 and C-5, the assignment of the ^{13}C n.m.r. spectrum of (IIe) is complete, and details are given in Table 4.

Fusicoccin Aglycone (IIc) *and Aglycone Tetra-acetate* (IId).—The spectra were compared with that of (IIc), and correlations were drawn on the basis of (*a*) expected substituent effects and (*b*) the line-shapes (residual second-order effects) in s.f.o.r.d. spectra. These were entirely consistent with the assignments made for (IIe).

Comparison between (IIc) and (IId) showed that acetylation of the primary alcohol function (C-19) produced the anticipated downfield shift of the α-carbon (C-19) signal and upfield shift of the β-carbon (C-15) signal. Acetylation of the secondary alcoholic function at C-12 produces a smaller α-shift and correspondingly smaller β-shifts upon C-11 and C-13. The effect upon C-11 is significantly smaller than upon C-13 (0.5 *cf.* 1.4 p.p.m.) and this appears to be a consequence of the fact that C-11 is ' quaternary ' in nature whereas C-13 is ' secondary '; we have characterised this phenomenon in other systems, and this work will be reported elsewhere. Esterification of the secondary alcoholic functions at C-8 and C-9 has little effect upon the chemical shifts of these nuclei, because the α- and β-effects tend to balance out. The C-7 signal shows the expected upfield β-shift. Table 4 demonstrates the inter-relationships between the spectra. In the spectrum of (IIc), the assignment of the C-6 and C-7 signals was based upon (*a*) the smaller shift expected for C-6 upon esterification of the C-8 hydroxy-group, and (*b*) the relative magnitudes of the residual splittings in the s.f.o.r.d. spectra of (IIc) and (IIe). In (IIe), the H-6 signal occurs at δ 3.00, and should not change position significantly in (IIc). The H-7 signal occurs at δ 2.20 in (IIe), and should be in a comparable position in (IIc). In (IIc) the residual splittings in the s.f.o.r.d. spectrum were 14.8 (δ_C 42.0 line) and 27.0 Hz (δ_C 41.8 line), respectively, in accord with the above assignment.

The olefinic carbon atoms C-2, C-10, and C-14 all showed significant shifts on going from (IIc) to (IId) or (IIe), *i.e.* upon esterification, and it was not possible to correlate the data. In an attempt to resolve this problem, small quantities of the lanthanide shift reagent $Eu(fod)_3$ were added to a solution of (IId) in $CDCl_3$. The total induced shifts after addition of five 5 mg portions are shown in Figure 2, for those carbon atoms whose

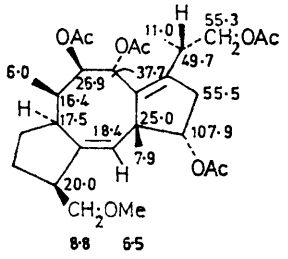


FIGURE 2 Pattern of lanthanide-induced shifts in (IId)

assignment is certain. In addition, the behaviour of the unassigned lines was as shown in Table 1.

TABLE 1

δ_C	Lanthanide-induced shift (Hz)
143.1	13.3
142.8	56.0
137.6	43.5
25.4	11.0
14.8	7.9

It may be deduced that the major site of complexation is the acetate function on C-12, followed by that on C-19. Complexation at the C-8 and C-9 acetate groups seems of only secondary importance, and co-ordination of the OCH_3 group is negligible. Examination of the shifts of the lines assigned collectively to C-2, C-10, and C-14 suggests that the δ_C 143.1 resonance may be assigned specifically to C-2. Comparison of (IId) with (IIc) suggests that there should be but little effect of acetylation upon C-14, but that C-10 should show the usual upfield (β) shift. The following assignments then result: C-14, δ_C 142.8 in (IId) and 142.3 in (IIc); C-10, 137.6 in (IId) and 140.1 in (IIc). This means that in (IIc), the C-2 signal occurs at 145.9 and upon acetylation undergoes an

upfield shift to 143.1 [in (IId)]. This is a surprisingly large effect when it is realised that C-2 is four bonds removed from the functions on C-12 and C-8; models suggest, however, that the conformation of the ring is such as to bring C-8 close to the 1,2-double bond.

Extrapolation of these results to (IIe) indicates that in this compound C-2 corresponds to the δ_C 144.2 line, C-14

TABLE 2

δ_C and off-resonance multiplicity	Assignment
99.3 (d)	C-1′
70.5 (d) (two lines)	Unidentified sugar resonances
69.8 (d)	
68.9 (d)	
61.1 (t)	C-6′
58.7 (q)	C-21

to 143.6 and C-10 to 138.0; the assignments of C-2 and C-14 may, however, be reversed.

Comparison of the lines assigned to C-18 and C-20 in these spectra gives no further information which can resolve the uncertainty between them. However, in all cases the s.f.o.r.d. spectra showed the quartet centred around δ_C 15 to be much sharper than that at δ_C 25; the former probably corresponds, then, to C-18, and the latter to C-20, as indicated in the previous discussion of (IIe).

TABLE 3

Assignment	Fusicoccin A triacetate (IIb) δ_C	Fusicoccin aglycone acetate (IId) δ_C
C-1	126.5	126.7
C-3	48.0	47.9
C-4	36.3	35.8
C-5	28.5	28.1
C-6	41.4	41.5
C-7	40.9	39.9
C-11	55.1	54.9
C-12	80.6	80.5
C-13	34.9	34.6
C-15	32.8	32.3
C-16	76.3	76.2
C-17	9.8	11.0
C-18	15.8	14.8
C-19	68.0	66.8
C-21	58.7	58.7

It was not possible to distinguish between the signals due to C-4 and C-5.

Fusicoccin A Triacetate (IIb).—Excluding acetate functions, the spectrum of (IIb) should consist of 32

TABLE 4

Assignment criteria †	Carbon	(IIe)	(IIc)	(IId)	(IIb)	[1-^{13}C]-Acetate *	[2-^{13}C]-Acetate *
A	1	127.4	125.8	126.7	126.5	+	
	2	144.2 or 143.6 or 138.0	145.9 or 142.3 or 140.1	143.1 or 142.8 or 137.6	144.1 or 140.1 or 139.1		+
A, B	3	47.6	48.1	47.9	48.0	+	
A, B, C	4	35.9 or 28.4	36.1 or 28.0	35.8 or 28.1	36.3 or 28.5	+	
A, B, C	5					+	
AB	6	41.4	41.8	41.5	41.4		+
A	7	40.7	42.0	39.9	40.9	+	
A, B	8	77.2	78.5	76.8	77.8		+
A, B	9	66.9	67.0	66.8	77.3	+	
	10	144.2 or 143.6 or 138.0	145.9 or 142.3 or 140.1	143.1 or 142.8 or 137.6	144.1 or 140.1 or 139.1		+
	11	56.8	55.4	54.9	55.1	+	
A, B	12	80.7	79.9	80.5	80.6		+
A, B	13	35.2	36.0	34.6	34.9	+	
	14	144.2 or 143.6 or 138.0	145.9 or 142.3 or 140.1	143.1 or 142.8 or 137.6	144.1 or 140.1 or 139.1		+
A	15	32.6	35.5	32.3	32.8	+	
A	16	76.5	76.5	76.2	76.3		+
A	17	11.4	9.6	11.0	9.8		+
B	18	15.3	14.3	14.8	15.8		+
A	19	67.4	64.4	66.8	68.0		+
B	20	24.9	24.3	24.5	24.9		+
A	21	58.8	58.7	58.7	58.7		
	22				114.5	+	
B	23				143.1		+
	24				75.6	+	
	25				25.8		+
	26				24.7		+
	1′				99.3		
	2′				70.5 or 69.8 or 68.9		
	3′						
	4′						
	5′						
	6′				61.1		

* + Indicates enrichment observed (five-fold enhancement of signal for [1-^{13}C] acetate, three-fold for [2-^{13}C] acetate.) † Assignments are all based initially upon the observed multiplicity in s.f.o.r.d. spectra. Where additional criteria are necessary, these are indicated by the code letters A, incremented heteronuclear decoupling; B, line-shape in s.f.o.r.d. spectra; C, biosynthetic arguments.

resonances, and 31 of these are observed. Thirteen resonances would be expected in the region which is characteristic of ^{13}C directly bonded to an oxygen function, and two of these appear to be coincident at δ_C 70.5. This region is very complex because of the six carbon atoms in the sugar residue which absorb here; these are not labelled during biosynthesis in the presence of either [1-^{13}C]- or [2-^{13}C]-acetate, since they are of non-acetate origin, and it is convenient to eliminate them from consideration. The methoxy-carbon atom C-21 is not enriched either, and these seven resonances are identified in Table 2.

The resonances due to the C-6′ side-chain were assigned from s.f.o.r.d. and undecoupled spectra. The C-22, C-23, and C-24 signals are instantly recognisable from their multiplicities and characteristic chemical shifts: the C-22 (δ_C 114.5) and C-23 (143.1) signals appear as a poorly defined triplet and doublet respectively, the latter being distinguished from the C-1 signal which is much more sharply defined. The C-24 signal is the lower aliphatic singlet, at δ_C 75.6.

The majority of resonances due to the aglycone carbon atoms were assigned by comparison with the results obtained for the aglycone derivatives. This is illustrated in Table 3.

The methyl carbon signals (C-20, C-25, and C-26) occur at δ_C 25.8, 24.9, and 24.7, respectively. In the s.f.o.r.d. spectrum, the quartet corresponding to the δ_C 24.9 line was significantly broader than the others, and is therefore assigned to C-20.

Individual assignment of the quaternary olefinic carbon signals is difficult, since considerable variations occur amongst the compounds studied; these probably arise from conformational differences between fusicoccin and its aglycone derivatives.

Detailed assignments are presented in Table 4, which also indicates the sites of enrichment in the two biosynthetic studies using [1-^{13}C]- and [2-^{13}C]-acetate.

EXPERIMENTAL

Proton homonuclear decoupling was performed at 100 MHz with a Varian HA-100 spectrometer, for a sample of (IIe) (30 mg) in $CDCl_3$ (0.4 ml).

Carbon-13 spectra were recorded at 25.16 MHz on a Varian XL-100-12/Varian Data Machines 16K 620-L spectrometer system; 8 K sampling points were used to give 4 K plotted data points after Fourier transformation. Samples were dissolved in 2 ml of $CDCl_3$ in a 10 mm n.m.r. tube, the solvent deuterium being used to provide a field-frequency lock. Interference from the $CDCl_3$ carbon signal made it preferable to record spectra of the two enriched samples of fusicoccin A triacetate in CCl_4, with the deuterium lock provided by an external D_2O sample contained in a concentric 5 mm n.m.r. tube.

Incorporation of [1-^{13}C]- *and* [2-^{13}C]-*Acetate into Fusicoccin.*—*Fusicoccum amydali* was grown in submerged culture in shaken flasks (4 l capacity; 1 l medium) as previously described.[3] After 48 h sodium [1-^{13}C]acetate (250 mg, 90 atom % excess and 254 mg, 88.2 atom % excess, respectively) was added aseptically to each of 2 flasks. The flasks were harvested after 5 days and the combined culture filtrate extracted with chloroform (3 × 750 ml). The combined extracts were evaporated and the residue acetylated at room temperature with pyridine–acetic anhydride. The acetylating reagents were removed under high vacuum and the residue was crystallised from light petroleum (b.p. 60—80°) yielding tri-*O*-acetylfusicoccin (IIb) (40 mg) as needles, m.p. 116—117°. The same procedure was followed with sodium [2-^{13}C]acetate (2 × 260 mg, 60 atom % excess ^{13}C).

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