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AN NMR STUDY OF THE LOSS OF CARBON-20 IN THE BIOSYNTHESIS OF GIBBERELLIN A_3 BY *GIBBERELLA FUJIKUROI*

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Key Word Index—*Gibberella fujikuroi*; resuspension cultures; biosynthesis; gibberellins.

Abstract—Resuspension cultures of *Gibberella fujikuroi*, strain GF-1a, were shown to metabolise potassium [3′-^{13}C]mevalonate to ^{13}C-enriched C_{19}-gibberellins, plus $^{13}CO_2$ (derived from the loss of carbon-20). The formation of [^{13}C]-gibberellins could be observed *in vivo* using ^{13}C NMR; however that of $^{13}CO_2$ could not. In contrast, removal of the mycelium and concentration of the filtrate at pH 12 enabled the $^{13}CO_2$ produced to be observed using ^{13}C NMR. During incubations of $H^{14}CO_2Na$ with this fungus, complete conversion to other radioactive products was observed, and the significance of these results in the light of previous work is discussed.

INTRODUCTION

The biogenesis of the biologically active C_{19}-gibberellins from their inactive C_{20}-analogues has been the subject of several investigations. Summarizing the available data it may be concluded that:

(i) Neither 1-, 5-, nor 9-protons are lost during the $C_{20} \rightarrow C_{19}$ transformation [1], thus excluding unsaturated intermediates involving these centres.

(ii) Both oxygen atoms in the lactone bridge of C_{19}-gibberellins are derived from the 19-oic acid of their C_{20}-precursors [2]. Thus the $C_{20} \rightarrow C_{19}$ conversion appears to involve an intermediate with an electrophilic centre at carbon-10 which is attacked by the 19-oic acid (therefore excluding a 10α-alcohol intermediate).

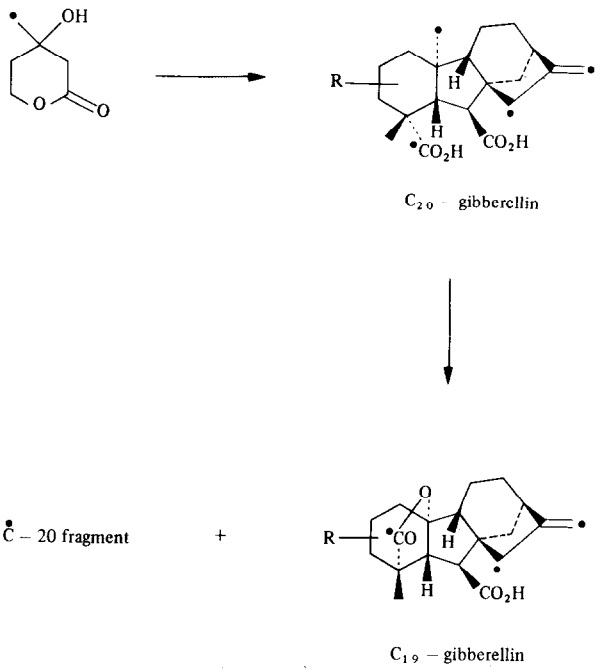
(iii) The ultimate fate of carbon-20 is, at least in part, as carbon dioxide [3, 4]. During incubations of kaurene (**1**, • = ^{14}C label), with *Gibberella fujikuroi* ACC917, Dockerill *et al.* [3, 4] isolated ^{14}C-labelled C_{19}-gibberellins. Based on the calculated amount of radioactivity lost by the release of only carbon-20, these workers were able to account for *ca* 80% as $^{14}CO_2$ in the gaseous phase of the cultures. However, despite analysis of the culture filtrates for $H^{14}CO_2H$ and $H_2{}^{14}CO$, the remaining 20% evaded detection, thereby inviting speculation that other intermediate C-20 fragment(s) might be involved.

The utility of NMR in biosynthetic investigations has already been established [5–10]. For example, the combination of NMR and $^2H/^{13}C$ enriched biosynthetic precursors, with suitable biological systems, has yielded much information on the coupling patterns of natural products. More recently, *in vivo* observation of metabolic conversions using ^{13}C NMR has been achieved by several groups [11–13]. Their results indicate that primary metabolic processes may often be observed at natural ^{13}C abundances, whereas secondary metabolic processes generally require ^{13}C enriched substrates.

Our aim was to investigate the application of ^{13}C NMR to the $C_{20} \rightarrow C_{19}$-gibberellin conversion, using the available [14, 15] [3′-^{13}C]mevalonolactone (**2**) as a source of [20-^{13}C]C_{20}-gibberellins. The transformation of **2** to C_{20}-(and thence to C_{19}-) gibberellins proceeds as summarized in Scheme 1, which traces the fate of the enriched carbon atoms. This conversion may be achieved in its entirety by use of the fungus *Gibberella fujikuroi*, of which strain GF-1a is known to be a fast and prolific producer of gibberellins [16].

RESULTS AND DISCUSSION

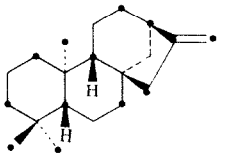
In preliminary experiments, utilising potassium [3′-^{13}C]mevalonate (**3**) as the gibberellin precursor, incubations containing either no substrate or radio-labelled



Scheme 1.

mevalonate with *G. fujikuroi* were performed under various conditions. The products were analysed by combined GC/MS or by TLC-radioscanning. The results confirmed that the conversion of mevalonate to C_{19}-gibberellins was more efficient in resuspension cultures than in non-resuspension cultures. Typical incorporations into GA_3 (**4**) from (3*R*)-mevalonolactone were 2.7% [cf. 17] and 16.0% [cf. 18] for non-resuspension and resuspension cultures respectively. In addition, various resuspension media were investigated with a view to reducing the carbon content of the cultures in the subsequent ^{13}C NMR experiments.

Thus, *G. fujikuroi* (after 5 days growth in 40% I.C.I. medium) was resuspended into one of several fresh media: distilled water, distilled water plus Mg^{2+} ions, distilled water plus trace elements, or distilled water plus Mg^{2+} ions and trace elements. GLC and GC/MS analysis of the metabolites present in the culture filtrates 4 days after resuspension showed that in each case the metabolites formed were those formed in a control culture using 0% I.C.I. medium. These metabolites were gibberellins A_3 (**4**) and A_{13} (**5**) and fujenal diacid (**9**) in the ratio 20:6:9, plus a small amount of 7β,18-dihydroxykaurenolide (**11**). Further, in each case the total concentration of these



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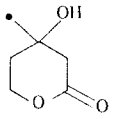

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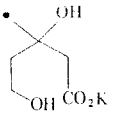

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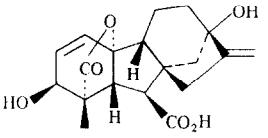

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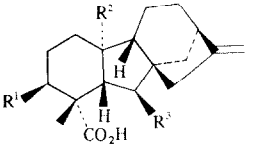
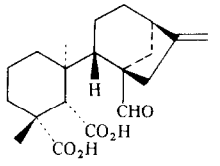


5 R^1 = OH, R^2 = CO_2H, R^3 = CO_2H
6 R^1 = H, R^2 = Me, R^3 = CO_2H
7 R^1 = H, R^2 = Me, R^3 = CHO
8 R^1 = OH, R^2 = Me, R^3 = CHO



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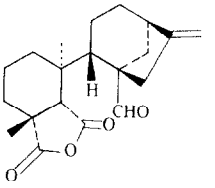

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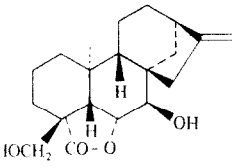

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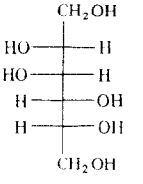
12 $CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2H$
13 $CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)CO_2H$
14 $CH_3(CH_2)_{16}CO_2H$
15 $CH_3(CH_2)_{14}CO_2H$



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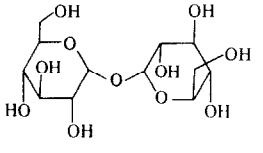

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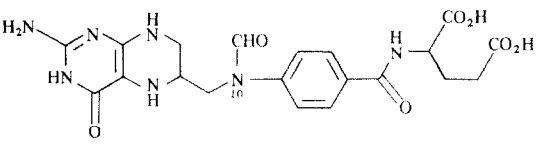

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metabolites was the same, and equal to 50% ± 10% of that in the control (0% I.C.I.) culture medium, as estimated by GLC of the MeTMSi derivatives. This difference is presumably due to the lack of glucose (as carbon source) in the cultures resuspended into distilled water.

Examination by GLC of a methanolic extract of *G. fujikuroi* mycelium at the time of resuspension indicated that < 1% of the usual metabolites were present. Thus the metabolites present 4 days later, in the distilled water cultures, appeared to have been formed biosynthetically from some intermediate retained in the mycelium upon resuspension. This was substantiated by the molecular ion cluster data (GC/MS) for gibberellin A_3 (MeTMSi) produced during incubations of potassium [3'-^{13}C]-mevalonate with distilled water resuspension cultures of *G. fujikuroi* (see below). These showed, typically, the following isotopic distributions: $^{13}C_0$ 53.2%, $^{13}C_1$ 12.3%, $^{13}C_2$ 18.9%, $^{13}C_3$ 15.7%, indicating dilution of the original ^{13}C label both by endogenous mevalonate (or its equivalent) and by some post-mevalonate gibberellin precursor. In addition, incubation of *G. fujikuroi*, resuspended in distilled water, had no qualitative or quantitative effect on the [^{3}H]-metabolites formed from [2-$^3H_2$]mevalonolactone after 4 days as compared with a 0% I.C.I. culture. Thus, the resuspension medium has no effect on the products formed from mevalonate when using this resuspension technique.

In subsequent incubations, therefore, resuspension into either distilled water or potassium phosphate buffer was generally used. The 4 day incubation of potassium [3'-^{13}C]mevalonate with *G. fujikuroi* under these conditions, with subsequent acidification to pH 2.5 and ethyl acetate extraction, yielded a sample which was examined by ^{13}C NMR and GC/MS. The ^{13}C NMR spectrum of the crude extract (as a C_5D_5N solution) is shown in Fig. 1, and the assignments for the observed signals are given in Table 1. These assignments were made by comparison with literature ^{13}C NMR data [19–31] and the previously unpublished ^{13}C NMR data shown in Table 2, and by investigation of the sample by GC/MS (as MeTMSi derivative).

In Fig. 1, ^{13}C natural abundance signals of all carbon atoms in gibberellin A_3 (**4**) are observable, as well as those ^{13}C-enriched carbons in both gibberellin A_3 and the other major terminal metabolites. Therefore, the predominant metabolites, including gibberellin A_3 (a C_{19}-gibberellin), were observable by ^{13}C NMR in extracts from culture filtrates of *G. fujikuroi* fed with potassium [3'-^{13}C]-mevalonate.

A time-course of incubations, performed and extracted in the same fashion, with harvesting of the culture filtrates at 8.5, 13, 22, 36, 48, and 96 hr after feeding of potassium [3'-^{13}C]mevalonate gave no extra information, since all spectra were qualitatively identical, showing only a gradual increase in intensity of each signal as a function of time. Repetition of this time-course experiment without extraction of the products, i.e. observing the intact cultures fed with ^{13}C-enriched substrate, appeared to be more promising and, indeed, after a 4 day incubation

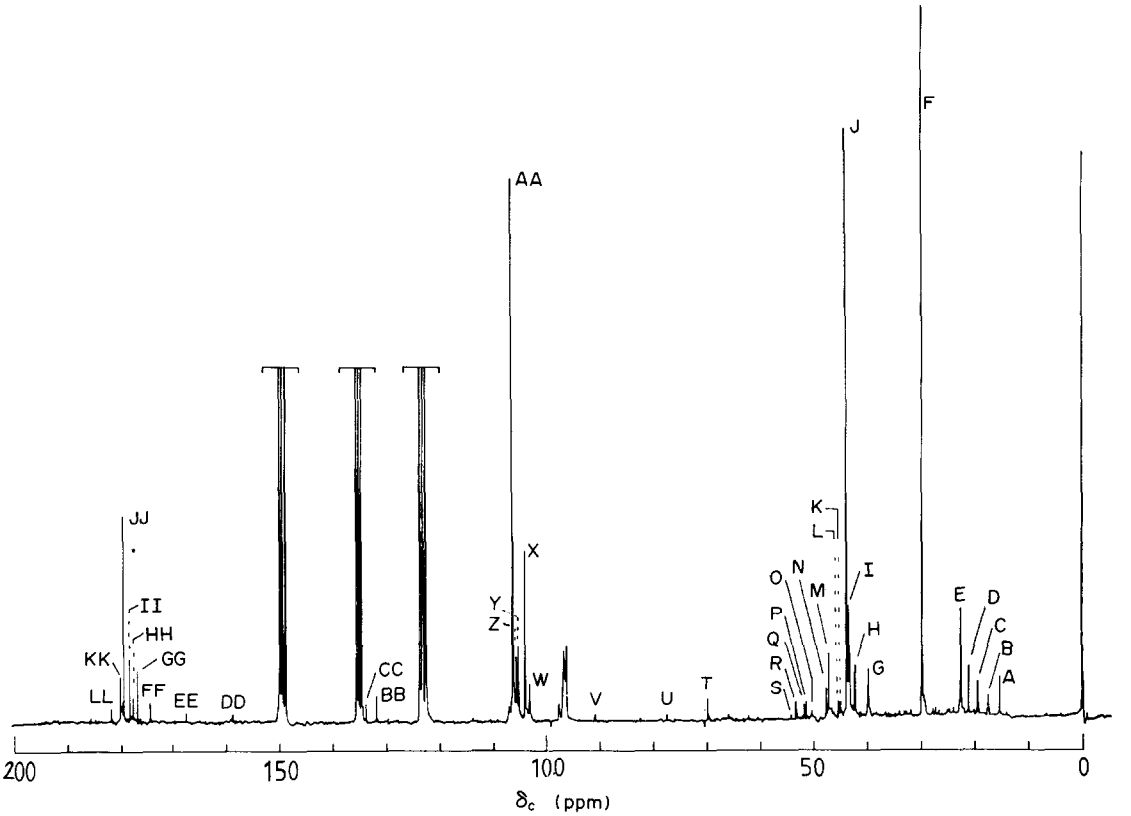


Fig. 1. ^{13}C NMR spectrum (50.1 MHz) of ethyl acetate-soluble products from incubation of potassium [3'-^{13}C]mevalonate with *G. fujikuroi*, (48 400 scans; solvent C_5D_5N, tube surrounded by CCl_4). Key: see Table 1.

Table 1. Assignments of signals in ^{13}C NMR spectrum shown in Fig. 1

Peak	δ_C	Assignment
A	15.6	C-18, u-GA_3
B	17.7	C-11, u-GA_3
C	19.7	x
D	21.3	C-20, e-7, 18$(OH)_2$kaurenolide?
E	22.7	C-20, e-fujenal diacid
F	29.8	Residual e-mevalonolactone
G	40.1	C-12, u-GA_3
H	42.5	C-15, e-7, 18$(OH)_2$kaurenolide?
I	43.7	C-15, e-fujenal diacid
J	44.1	C-15, e-GA_3
K	45.3	x
L	45.6	C-14, u-GA_3
M	47.5	C-15, e-GA_{13}
N	47.9	x
O	50.6	C-8
P	51.7	C-5
Q	52.1	C-6
R	53.6	C-9
S	54.5	C-14
T	70.0	C-3
U	77.7	C-13
V	91.1	C-10
		u-GA_3
W	103.7	x
X	104.4	C-17, e-fujenal diacid
Y	105.6	C-17, e-GA_{13}
Z	106.1	C-17, e-7, 18$(OH)_2$kaurenolide?
AA	106.7	C-17, e-GA_3
BB	132.3	C-1, u-GA_3
CC	134.4	C-2, u-GA_3
DD	159.1	C-16, u-GA_3
EE	168.1	x
FF	174.9	C-7, u-GA_3
GG	178.0	C-19, e-GA_{13}
HH	178.3	x
II	178.6	C-20, e-GA_{13}
JJ	179.8	C-19, e-GA_3
KK	180.4	x
LL	182.2	C-19, e-7, 18$(OH)_2$kaurenolide?

Key: u, unenriched; e, ^{13}C-enriched; x, unidentified.

period, ^{13}C-enriched gibberellins could be detected in such cultures (see Figs 2 and 3). Their concentrations were, however, low and the signal intensities correspondingly small. Enriched products are marked with an asterisk (*) in Fig. 3. In addition, many much stronger signals due to the mycelial components were observed. Chloroform extraction of 9 day old *G. fujikuroi* mycelium revealed that the major mycelial components were fatty acid triglycerides [32]. Saponification of this extract followed by analysis of the methyl esters by capillary GLC showed the major constituent fatty acids to be as indicated in Table 3.

The assignments for the NMR spectrum shown in Fig. 2 are given in Table 4, from which it can be seen that a monosaccharide, mannitol (**16**) was also tentatively identified [33]. This compound has previously been isolated from *G. fujikuroi* by Borrow *et al.* [34]. Interestingly, examination of mycelia alone (grown in 40% I.C.I. medium, then harvested and resuspended into D_2O in the NMR tube) showed that in cultures between 1 and 9 days old, the mannitol concentration decreased until no longer detectable. Over the period from *ca* 9 days to at least 6 weeks, a new saccharide accumulated. Figure 4 shows part of the spectrum from 6 week old *G. fujikuroi* mycelium, the remainder being identical to the spectrum shown in Fig. 2, except that no ^{13}C-enriched mevalonate was present. The second saccharide was identified as α,α-trehalose (**17**) by addition of an authentic sample, and this compound has subsequently been detected in methanolic mycelial extracts from *G. fujikuroi* by GC/MS [Gaskin, P., personal communication].

Interestingly, trehalose is present after 4 days in mycelium resuspended into distilled water (cf. Fig. 3), showing that primary metabolism continues even though no glucose is present in the culture medium. This sugar has previously been detected by ^{13}C NMR during incubations of *Propionibacterium shermanii* [11] (in a glucose-supplemented medium) and in encysted *Acanthamoeba castellanii* [35].

Filtration of potassium [3′-^{13}C]mevalonate fed cultures removed almost all of the mycelial signals (from the spectrum of the filtrate), although the ^{13}C-enriched metabolites were still detectable. Hence this technique was used to investigate the $C_{20} \rightarrow C_{19}$-gibberellin conversion, since it also enabled concentration of the culture filtrate (under reduced pressure), which effectively increased the sensitivity of the NMR experiment.

Feeding experiments were subsequently performed as previously described, using resuspension cultures of *G. fujikuroi* with potassium [3′-^{13}C]mevalonate as substrate. However, in an effort to 'trap' any $^{13}CO_2$, $H^{13}CO_2H$ etc. formed by the loss of carbon-20 during the biosynthesis of gibberellin A_3 (**4**), these incubations were buffered at pH 10. After 4 days, the mycelia were filtered off and examined by NMR, although no extra signals were observed (cf. Fig. 2, minus substrate). The culture filtrates were adjusted to pH 12 and concentrated to dryness under reduced pressure. The residue was subsequently dissolved in D_2O–dioxan and examined by ^{13}C NMR. The spectrum from one such experiment is shown in Fig. 5. This experiment was repeated several times with qualitatively similar results.

It may be seen from Fig. 5 that the chemical shifts of the ^{13}C-enriched metabolites are not the same as those in the spectrum shown in Fig. 1. This is partly because of the change of solvent (D_2O cf. C_5D_5N) but also because, at the higher pH used, gibberellin A_3 (**4**) is converted to the isolactone (**18**), which is then ring-opened, by the known sequence [36, 37] shown in Scheme 2. The presence of compounds **4**, **18** and **19** was confirmed by GC/MS analysis of the concentrate. Thus, the qualitative metabolism of the substrate (**3**) by the fungus was not significantly altered by the change in pH of the culture.

More significant is the presence of the signal at δ168.9, characteristic of carbonate, CO_3^{2-}, as confirmed by re-recording of the spectrum after addition of authentic material. In control experiments, with either unlabelled or no mevalonate present, this signal was always present at a level of 20–30% of that seen in Fig. 5, by comparison of the peak integrals of the CO_3^{2-} and dioxan signals. The source of this carbonate may be the other biosynthetic processes in the fungus (of which there are many); alternatively this signal may represent CO_2 absorbed from the atmosphere during the incubation or work-up. In any case, 70–80% of the CO_3^{2-} was produced as a direct consequence of the metabolism of ^{13}C-enriched

Table 2. ^{13}C NMR spectra of some gibberellins and kaurenoids (δ_C from internal TMS)

Carbon No.	Compound						
	9[a]	**9**[b]	**10**[a]	**10**[b]	**6**[b]	**7**[b]	**8**[b]
1	37.3 (*t*)[c]	37.9 (CH_2)[d]	33.2 (CH_2)[d]	33.0 (CH_2)[d]	40.4 (*t*)[c]	40.4 (*t*)[c]	28.6
2	19.6 (*t*)	20.0 (CH_2)	19.6 (CH_2)	19.6 (CH_2)	20.6 (*t*)	20.6 (*t*)	34.6
3	36.2 (*t*)	37.1 (CH_2)[br]	32.0 (CH_2)	32.3 (CH_2)	39.5 (*t*)	38.2 (*t*)	70.6
4	44.8 (*s*)	45.1[br]	44.5	44.8	44.6 (*s*)[e]	45.2 (*s*)[e]	x
5	60.1 (*d*)	60.6 (CH)[br]	56.1 (CH)	56.1 (CH)	57.7 (*d*)[f]	58.4 (*d*)[f]	50.5
6	178.1 (*s*)	177.3[br]	172.4	172.9	52.3 (*d*)	58.2 (*d*)[f]	58.2[e]
7	207.6 (*d*)	204.9 (CH)[br]	203.8	203.8	178.0 (*s*)	205.7 (*d*)	207.2
8	59.3 (*s*)	59.3[br]	58.9	59.0	49.9 (*s*)	50.3 (*s*)	x
9	47.9 (*d*)	48.4 (CH)[br]	46.3 (CH)	46.7 (CH)	57.4 (*d*)[f]	57.2 (*d*)	58.3[e]
10	43.1 (*s*)	43.0	41.8	42.0	45.0 (*s*)[e]	44.4 (*s*)[e]	45.3
11	19.2 (*t*)	19.8 (CH_2)	17.8 (CH_2)	17.9 (CH_2)	17.3 (*t*)	17.4 (*t*)	17.5
12	31.8 (*t*)	32.5 (CH_2)[br]	28.8 (CH_2)[e]	28.7 (CH_2)[e]	32.5 (*t*)	32.2 (*t*)	32.3
13	43.9 (*d*)	44.4 (CH)	43.9 (CH)	44.3 (CH)	40.6 (*d*)	38.5 (*d*)	38.4[f]
14	32.8 (*t*)	33.1 (CH_2)	32.4 (CH_2)[e]	32.6 (CH_2)[e]	38.8 (*t*)	37.3 (*t*)	37.4[f]
15	43.3 (*t*)	43.7 (CH_2)	42.7 (CH_2)	42.9 (CH_2)	47.1 (*t*)	43.6 (*t*)	43.5
16	150.9 (*s*)	152.6	150.9	151.8	157.5 (*s*)	157.6 (*s*)	157.8
17	105.0 (*t*)	104.5 (CH_2)	105.1 (CH_2)	104.9 (CH_2)	105.8 (*t*)	106.6 (*t*)	106.4
18	29.1 (*q*)	30.4 (CH_3)	29.8 (CH_3)	29.3 (CH_3)	30.1 (*q*)	30.4 (*q*)	24.6
19	181.0 (*s*)	179.5[br]	175.9	176.7	180.4 (*s*)	179.5 (*s*)	x
20	21.7 (*q*)	21.9 (CH_3)	22.6 (CH_3)	22.9 (CH_3)	15.7 (*q*)	15.8 (*q*)	15.8

Key: a = in $CDCl_3$; b = in C_5D_5N; c = assigned by off-resonance decoupling; d = assigned by DEPT; e, f = resonances may be interchanged in this column; br = resonance broad; x = resonance not observed.

Table 3. Fatty acids identified in saponified chloroform extract from 9-day-old *G. fujikuroi* mycelium

Fatty acid	Abundance % of total
Oleic acid (**12**)	38
Linoleic acid (**13**)	22
Stearic acid (**14**)	8
Palmitic acid (**15**)	32

mevalonate, presumably via $^{13}CO_2$. No signal for $H^{13}CO_2Na$ (expected at δ171.5 under these conditions) was observed during these experiments.

To investigate the possibility of using ^{13}C NMR to detect formate as an intermediate during the $C_{20} \rightarrow C_{19}$ conversion, the methodology developed above was applied to incubations of *G. fujikuroi* fed with $H^{13}CO_2Na$ (at pH 10). After 4 days, the mycelium was harvested and the ^{13}C NMR spectrum recorded as before. However, no signals other than those present in a 'blank' mycelial sample were observed. Surprisingly, the culture filtrate produced a spectrum which was almost totally featureless. Therefore, although the $H^{13}CO_2Na$ had been metabolised by the fungus, any products other than CO_2 were not detectable by ^{13}C NMR. At this point recourse to ^{14}C-labelled substrates was made.

Table 4. Assignments for ^{13}C NMR spectrum shown in Fig. 2

Peak	δ_C	Assignment
A	14.8	Terminal Me in fatty acid triglycerides
B	23.5	C-17 in O, L, S + C-15 in P
C	25.6	C-3 in O, L, S, P
D	26.3	C-11 in L
E	26.9	Me in ^{13}C-enriched mevalonate $^-K^+$
F	27.9	C-8 + C-11 in O; C-8 + C-14 in L
G	30.0	C-4, 5, 6, 7, 12, 13, 14, 15 in O;
H	30.2	C-4, 5, 6, 7, 15 in L;
I	30.6	C-4 → C-15 in S; C-4 → C-13 in P
J	32.3	C-16 in L
K	32.8	C-16 in O, S + C-14 in P
L	34.4	C-2 in O, L, S, P
M	62.6	C-1 + C-3 in glyceride carbons
N	63.9	C-1 + C-6 in mannitol
O	64.1	x
P	69.7	C-2 in glyceride carbons
Q	70.3	C-3 + C-4 in mannitol
R	71.2	x
S	71.8	C-2 + C-5 in mannitol
T	128.7	C-10 + C-12 in L
U	130.4	C-9 + C-10 in O; C-9 + C-13 in L
V	172.2	C-1 in O, L, S, P triglycerides
W	172.4	

Key: x = unidentified; O = oleic acid (**12**); L = linoleic acid (**13**); S = stearic acid (**14**); P = palmitic acid (**15**), all as triglycerides.

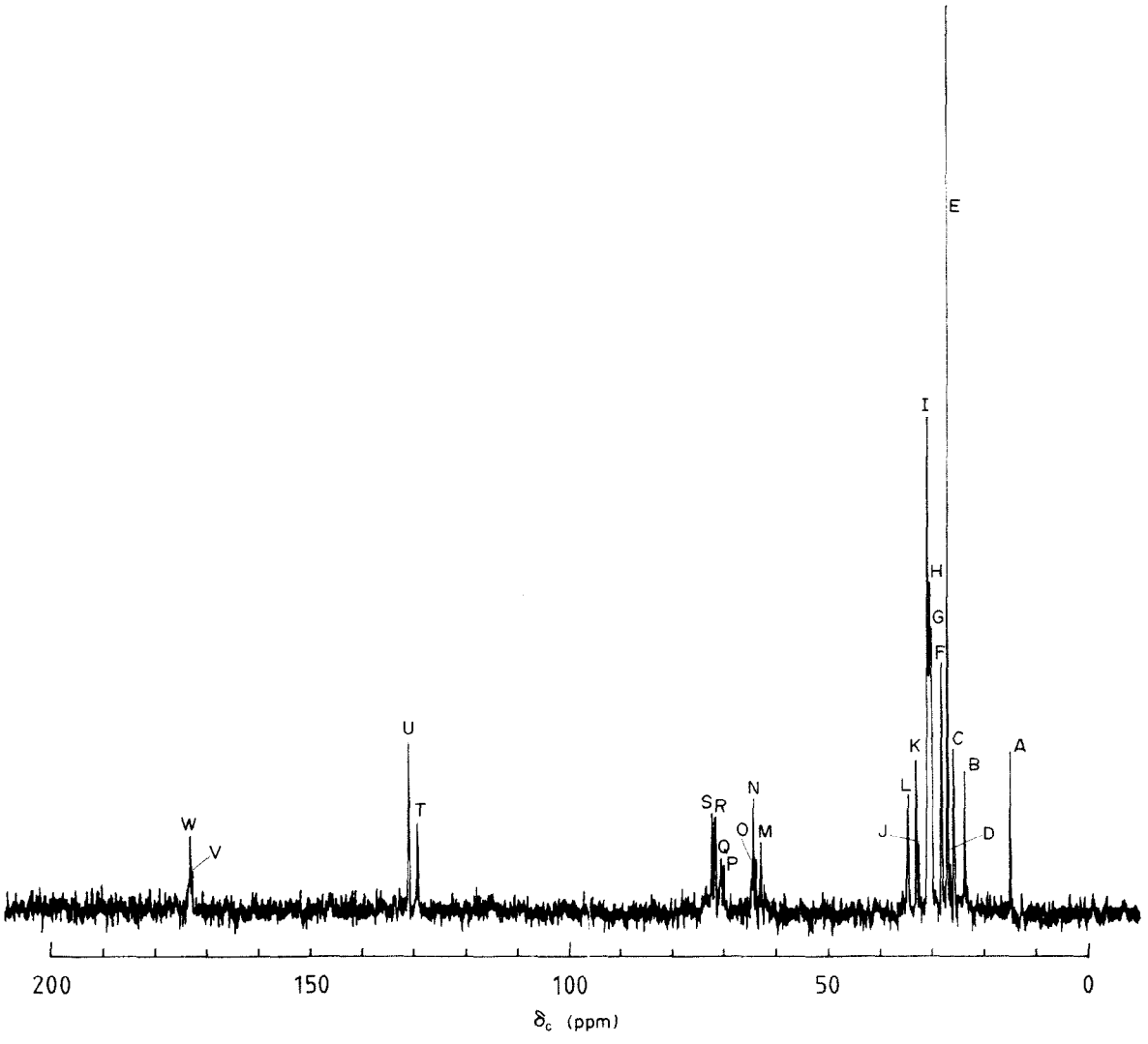


Fig. 2. ^{13}C NMR spectrum (50.1 MHz) of whole culture of *G. fujikuroi* plus potassium [3′-^{13}C]mevalonate (1.54 mM) at time $t = 0$, (10000 scans). Key: see Table 4.

Incubations of $H^{14}CO_2Na$ with *G. fujikuroi* under identical conditions to those used previously (resuspension into potassium phosphate buffer at pH 10, 4 day incubation) and subsequent filtration of the mycelium showed the fate of the original radioactivity to be as indicated in Table 5. These data show that formate is converted to a volatile product (presumed to be CO_2) plus some mycelial components. Attempted extraction of these latter components was unsuccessful with both methanol (15% of mycelial radioactivity extractable) and chloroform (*ca* 1%). That the conversion of formate was not merely due to aerial oxidation is shown by the control experiments with no fungus present, when 99% recovery of radioactivity in the aqueous fraction was realised. The most reasonable interpretation of these data is that the CO_2 formed by fungal assimilation of HCO_2Na is lost from solution by exchange with atmospheric CO_2 and indeed, as shown in Table 5, this is the outcome when $Na_2{}^{14}CO_3$ is shaken with or without fungus (all other conditions being the same).

In conclusion it appears that *G. fujikuroi* transforms formate to a mixture of CO_2 (exchanged with atmospheric CO_2 during the incubation) plus, as yet unidentified, mycelial components. Enzyme systems (formate dehydrogenases) capable of such transformations have been isolated from many microbes [38] and intact plants [39]. The mycelial products are almost certainly C_1-pool compounds formed via some C_1-transfer species such as N^{10}-formyltetrahydrofolate (**20**). The latter is known to be involved in the biosynthesis of S-adenosylmethionine, and thus the methylation of a wide range of polyketides etc. Therefore the non-detection by ^{13}C NMR of the $H^{13}CO_2Na$ derived products is, perhaps, understandable.

Our data show that if formate is indeed a transient intermediate formed after the loss of carbon-20 from a C_{20}-gibberellin, it is likely that part of this will be converted to carbon dioxide and part to C_1-pool products not previously examined. These observations may provide the reason why Dockerill *et al.* [3, 4] failed to account for

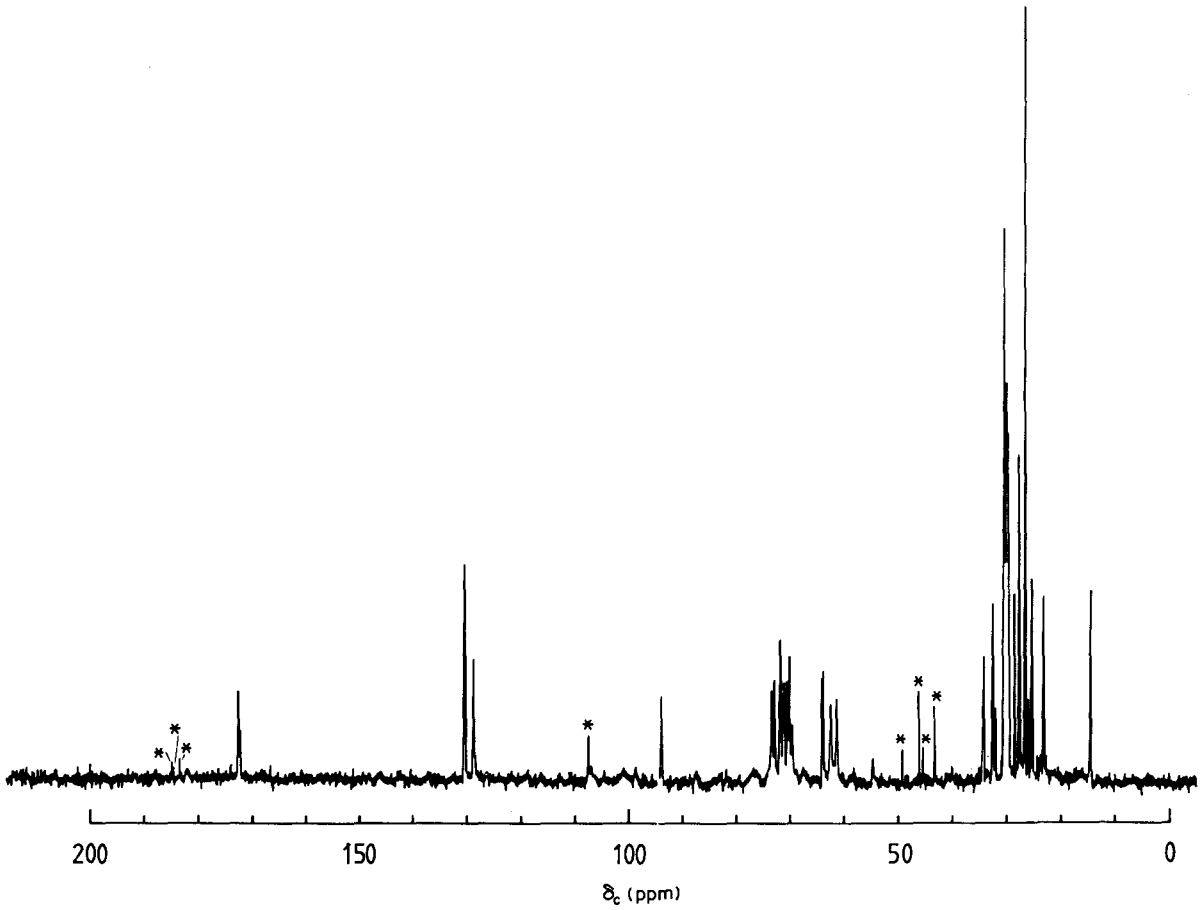


Fig. 3. ^{13}C NMR spectrum (50.1 MHz) of whole culture of *G. fujikuroi* incubated with potassium [3'-^{13}C]mevalonate (1.54 mM) after 4 days (63 500 scans). Key: ^{13}C-enriched products indicated by *.

100% of the carbon-20 derivable radioactivity during their incubations of [20-^{14}C]kaurene (**1**) with *G. fujikuroi*.

EXPERIMENTAL

Fungal work—general. The wild-type strain, GF-1a, of *Gibberella fujikuroi* was used for all fungal work described. The media used for general incubations contained either 40% or 0% of the inorganic nitrogen (NH_4NO_3) in the medium described by the I.C.I. group of Borrow *et al.* [32]. Resuspension cultures were prepared as follows. The fungus was grown in 40% I.C.I. medium for the appropriate period (usually 4 days) then the mycelium was filtered off under vacuum and washed with sterile distilled water. The mycelium was transferred to a fresh shake flask containing resuspension medium: 0% I.C.I. medium or other as appropriate.

Resuspension media. Alternative resuspension media consisted of either distilled water or one of two phosphate buffers: (a) aq. KH_2PO_4 (37 mM, pH 4.8), or (b) aq. K_2HPO_4 (37 mM, pH adjusted to 10.0 using 5 M KOH). When examining incorporations into gibberellin A_3, the method of isotope dilution analysis was used, as described in ref. [18].

Analysis of culture filtrates. The pH of each culture was measured, then adjusted to pH 2.5 using 2 M HCl (for samples not to be examined by NMR). The soln was extracted using EtOAc (3 equal vols) and the extract washed with H_2O (1 vol.), and concd under red. pres. at a temp. below 70°. An aliquot of the concentrate was dissolved in MeOH and treated with ethereal CH_2N_2, then concd to dryness in a stream of N_2. Samples were trimethylsilylated using Sweeley's reagent (hexamethyldisilazane–trimethylchlorosilane–pyridine, 3:3:2) for packed column GLC, or *N*-methyl, *N*-trimethylsilytrifluoroacetamide for capillary GLC and GC/MS.

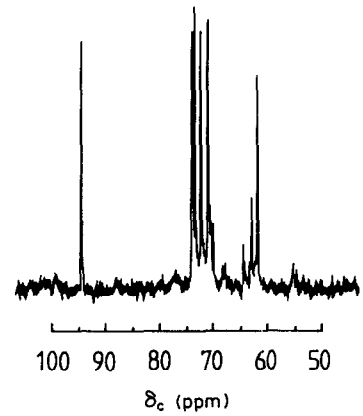


Fig. 4. Portion of 50.1 MHz ^{13}C NMR spectrum of *G. fujikuroi* mycelium aged 6 weeks (4000 scans).

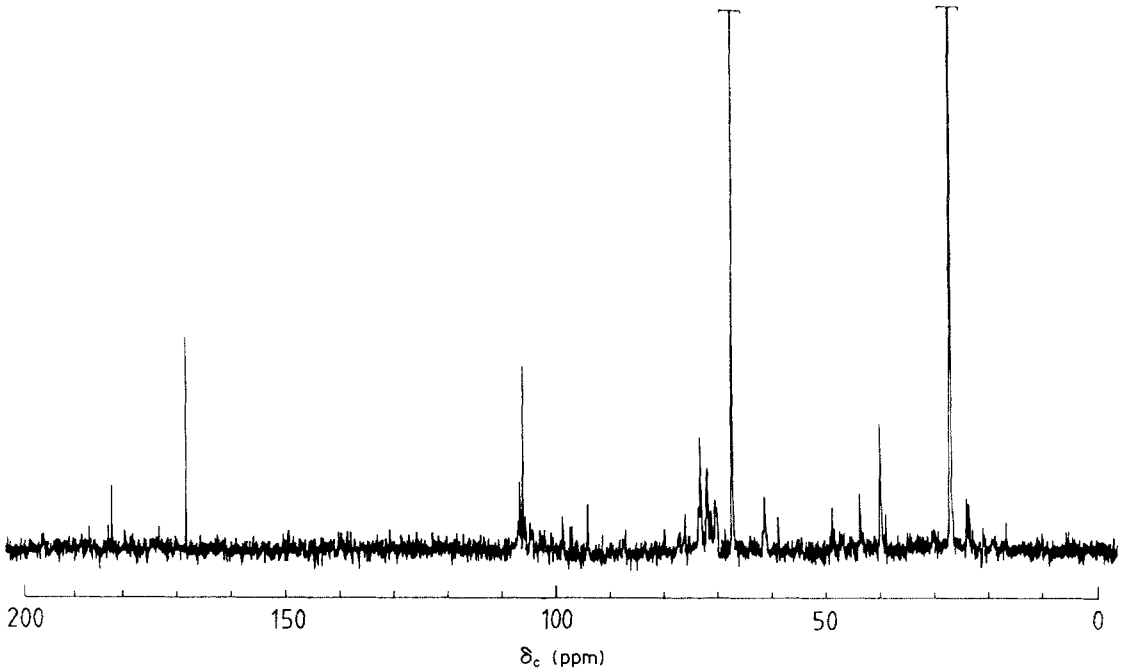
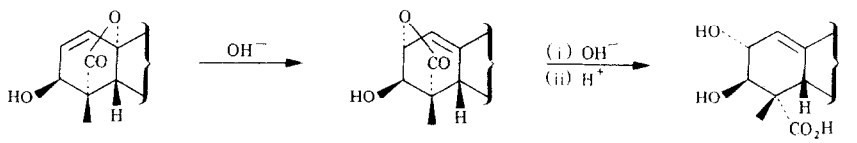


Fig. 5. ^{13}C NMR spectrum (50.1 MHz) of concentrated culture filtrate from 4 day incubation of potassium [3'-^{13}C]mevalonate with *G. fujikuroi* at pH 10 (54 500 scans).



4 18 19

Scheme 2.

Table 5. Distribution of radioactivity during incubations of $H^{14}CO_2Na$ and $Na_2{}^{14}CO_3$ with and without *G. fujikuroi* present

Substrate	Fate (% of original radioactivity)		
	Aqueous	Mycelial	Unaccounted for
$H^{14}CO_2Na$	5	45	50
$H^{14}CO_2Na$ (no fungus)	99	0	1
$Na_2{}^{14}CO_3$	3	0	97
$Na_2{}^{14}CO_3$ (no fungus)	2.5	0	97.5

General technique for mycelial NMR study. A 250 ml shake flask containing 40% I.C.I. medium (50 ml) was inoculated with *G. fujikuroi* (1 ml) and allowed to grow for the required period of time, whilst shaken on an orbital shaker at 200 rpm and 27°. Subsequently, the mycelium was filtered under vacuum and washed with deionized H_2O (3 × 50 ml), then halved and gently mixed with D_2O (2 ml). The resulting sample was placed in an NMR tube (10 mm o.d.) to a depth of *ca* 6 cm and examined immediately by ^{13}C NMR.

General technique for examination of culture filtrates by ^{13}C NMR. After the appropriate incubation time, the mycelium was filtered off under vacuum. The filtrate was adjusted to pH 12 using 5 M KOH. The soln was concd to dryness under red. pres., then dissolved in D_2O–dioxan (150:1, 1.5 ml) and centrifuged (2000 rpm, 10 min). The supernatant was then examined by ^{13}C NMR. ^{13}C NMR spectra were recorded using a Jeol FX-200 Fourier transform spectrometer, with chemical shifts quoted in ppm relative to external tetramethylsilane (via internal dioxan at 67.4 ppm). Accumulation parameters: 16 384 data points, 652 msec acquisition time, 200 msec pulse delay, 10 μsec (*ca* 37°) pulse width.

Assignments of ^{13}C NMR spectra in Table 2. Spectra reported in Table 2 were assigned with the aid of either off-resonance decoupled or DEPT [40] techniques. Where determined by the former method multiplicities are designated (*q*), (*t*), (*d*), or (*s*), and where the DEPT technique was used, the designations (CH_3), (CH_2) or (CH) are used.

Substrate concentrations. Potassium [3'-^{13}C]mevalonate, 89.0 atom % ^{13}C (generated from the lactone form by treatment with 2 mol equivalents of aq. KOH at 30° for 30 min) was generally used at a concn of 5.0 mg (3*R*, *S*)-mixture per 50 ml incubation. Sodium [^{13}C]formate, 99.8 atom % ^{13}C, was used at a concentration of 1.0 mg per 50 ml incubation. Sodium [^{14}C]formate and sodium [^{14}C]carbonate (specific activities 2.18 and 2.06 GBq/mmol respectively) were incubated at concns of 24 kBq and 20.5 kBq per 50 ml, respectively.

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