Studies in Terpenoid Biosynthesis. Part XIII.[1] The Biosynthetic Relationship of the Gibberellins in *Gibberella fujikuroi*

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A comparison of the results of feeding *ent*-7-hydroxygibberell-16-en-19-oic acid, *ent*-7-oxogibberell-16-en-19-oic acid, and *ent*-gibberell-16-ene-7,19-dioic acid (gibberellin A_{12}), in terms of the distribution of mevalonate, to *Gibberella fujikuroi*, reveals that there is a divergence between the pathways leading to the 3-hydroxylated and the non-3-hydroxylated gibberellins at the C-7 aldehyde stage. The distribution of label between the C_{19} and C_{20} gibberellins after 6 and 24 h incubations suggests that the free C_{20} gibberellins (A_{14}, A_{37}, A_{36}, and A_{13} or A_{15}, A_{24}, and A_{25}) may not lie on the direct pathway to the C_{19} gibberellins. Gibberellins A_4 and A_7 are transformed into gibberellic acid. Incubation of gibberellin A_{12} with *G. fujikuroi* may be used to prepare gibberellins A_{24} and A_{25}.

THE gibberellin plant growth hormones possess a unique carbon skeleton and differ from one another in their oxygenation patterns. Some aspects of their biosynthetic inter-relationships within the fungus *Gibberella fujikuroi* (ACC 917) form the subject of this paper. In our previous work [2] on the sequence of oxidation of ring B of the kauranoid precursors of the gibberellins, we established that *ent*-7α-hydroxykaur-16-en-19-oic acid (1) was converted into the gibbane aldehyde (3) and that it formed the point of divergence between the gibberellin and kaurenolide pathways. Both the gibbane alcohol (2) [3] and the aldehyde (3) [4] have been shown to be efficient precursors of gibberellic acid (6). Based on comparative levels of incorporation Cross suggested [4] that 3-hydroxylation of the aldehyde (3) preceded further oxidation at C-7. MacMillan has recently shown [5] with a mutant strain of *G. fujikuroi*, that 3-hydroxylation of the aldehyde (3) and the formation of gibberellins A_{14} (7), A_4 (8), and A_7 (5) occurs under conditions in which gibberellin A_{12} (4) is not metabolised. While our work was in progress, MacMillan also described [6] results of feeding substrates which occur beyond *ent*-kaur-16-en-19-oic acid on the pathway, to another mutant which was blocked for gibberellin biosynthesis between *ent*-kaur-16-en-19-al and *ent*-kaur-16-en-19-oic acid and which therefore lacked endogenous metabolites. In this mutant gibberellin A_{12} alcohol (2), the aldehyde (3), and gibberellin A_{14} aldehyde were efficient precursors of the ring-A-hydroxylated gibberellins A_1 (9), A_3 (6), A_4 (8), A_7 (5), A_{13} (11), and A_{36} (15) whilst gibberellin A_{12} (4) was incorporated into the non-3-hydroxylated gibberellins A_9 (16), A_{15} (12), A_{24} (14), and A_{25} (10). Our results are based on comparative feeding of *ent*-7-hydroxygibberell-16-en-19-oic acid (gibberellin A_{12} alcohol) (2), *ent*-7-oxogibberell-16-en-19-oic acid (gibberellin A_{12} aldehyde) (3), and *ent*-gibberell-16-ene-7,19-dioic acid (gibberellin A_{12}) (4) to a normal strain of *G. fujikuroi* in which the added substrates represented a small fraction of the total mass of gibberellin production. Hence there was less likelihood of our observing induced pathways. However the overall results are in substantial agreement with the mutant studies. They are in contrast to recent work [7] with a higher plant system derived from *Pisum sativum* in which gibberellin A_{14} was hydroxylated at C-13 prior to oxidation of C-20.

The [17-^{14}C]-substrates were fed to three-day-old cultures of *G. fujikuroi* which were then grown for a further 6 and 24 h. 7-Oxogibberell-16-en-19-oic acid (3) was shown to be unstable under these conditions. It underwent autoxidation to give, amongst other products, gibberellin A_{12}. This fermentation was therefore harvested after 3 h. The normal pattern of gibberellin production of this strain was established by using [2-^{14}C]mevalonic acid. This formed a basis from which conclusions could be drawn on the relative proportions of the metabolites which were formed. The gibberellins were separated by chromatography on Sephadex LH 20 in the biphasic system light petroleum–ethyl acetate–acetic acid–methanol–water (100 : 80 : 5 : 40 : 7) [8] and then the individual fractions were further purified and identified by radio-t.l.c. scanning against authentic samples. To provide sufficient mass for additional identification, the 6 h fermentation with the alcohol (2) was repeated and the product was diluted with the crude extract (270 mg) from 2·5 l of a 3·5-day-old *G. fujikuroi* fermentation. The results are tabulated.

Gibberellin A_1 was not observed in this culture. The 'unknown fraction' contained at least four minor components and was eluted in the region associated with gibberellin A_{36}.[8] One component was tentatively identified by the mass spectrum of its methyl ester as gibberellin A_{16}. However the components could not be separated sufficiently cleanly for counting purposes. An interesting feature of the mevalonate experiment was the recovery of labelled *ent*-kaurene and *ent*-7α-hydroxykaurenoic acid as well as a group of gibberellins

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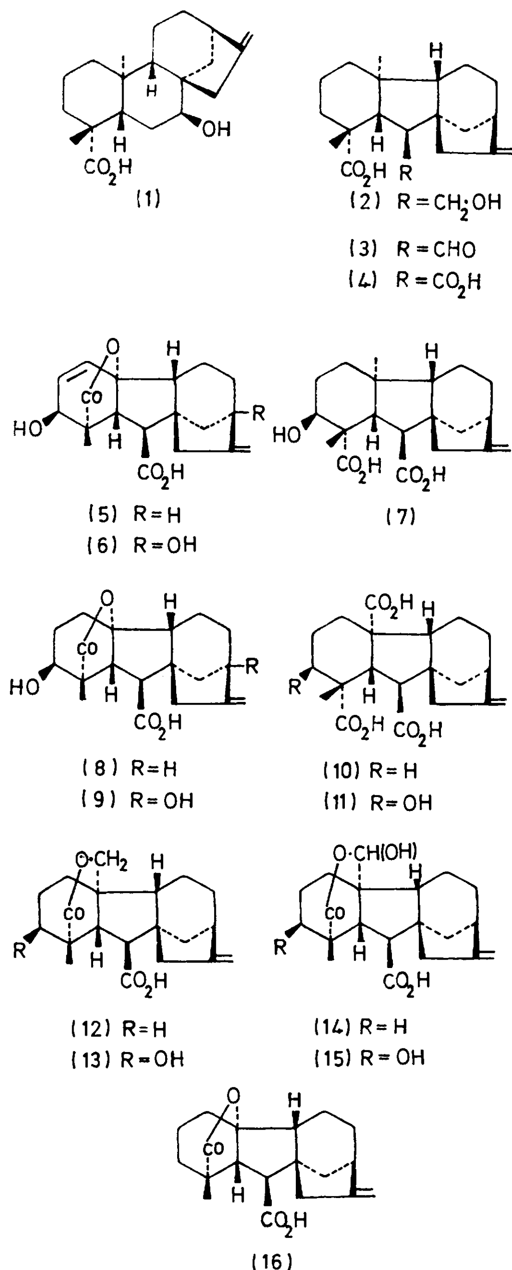
which do not appear to be further metabolised readily by this culture.

A number of conclusions may be drawn from these results. Gibberellin A_{12} was metabolised in a different manner to the alcohol (2) and the aldehyde (3) and was converted efficiently and rapidly into the non-3-hydroxylated gibberellins A_9 (16), A_{15} (12), A_{24} (14), and A_{25} (10). After 6 h 75% and after 24 h 67% of the radioactivity which was recovered was in these four compounds. MacMillan observed [6] some conversion of gibberellin



A_{12} into 3-hydroxylated gibberellins in the blocked mutant. Since these experiments were carried out in the absence of the normal range of substrates, it is arguable that some of this might represent a microbiological transformation. Although in a preliminary experiment we showed that gibberellin A_9 (16) was

TABLE 1

[2-^{14}C]Mevalonic acid incubation

Metabolite	Disint. min^{-1}	Radioactivity recovered (%)
ent-Kaurene	2.11×10^6	9·9
ent-7α-Hydroxykaurenoic acid	5.48×10^5	2·6
Gibberellin A_9	2.27×10^5	1·1
Gibberellin A_{37}	9.10×10^5	4·3
Gibberellin A_{14}	2.78×10^5	1·3
Gibberellins A_4 and A_7	Not detected	
Gibberellin A_{13}	1.11×10^6	5·2
Gibberellin A_3	3.87×10^6	18·1

TABLE 2

ent-7-Hydroxy[17-^{14}C]gibberell-16-en-19-oic acid incubations

Metabolites (in order of elution)	6 h Disint. min^{-1}	6 h Radioactivity recovered (%)	24 h Disint. min^{-1}	24 h Radioactivity recovered (%)
Gibberellin A_9	2.78×10^5	1·3	2.64×10^5	1·2
Gibberellin A_{15}	1.11×10^5	0·5	9.73×10^4	0·41
Gibberellin A_{24}	4.17×10^5	1·94	4.85×10^5	2·03
Gibberellin A_4	1.91×10^6	8·9	2.32×10^5	0·97
Gibberellin A_7	1.45×10^6	6·7	8.3×10^4	0·35
Gibberellin A_{37}	2.89×10^6	13·5	1.72×10^6	7·2
Gibberellin A_{25}	6.68×10^5	3·1	4.83×10^5	2·02
Gibberellin A_{14}	2.70×10^6	12·6	2.21×10^6	9·3
' Unknowns '	1.07×10^6	5·0	1.57×10^6	6·6
Gibberellin A_{13}	1.44×10^6	6·7	1.90×10^6	7·8
Gibberellin A_3	4.64×10^6	21·6	9.50×10^6	39·8

TABLE 3

ent-7-Oxo[17-^{14}C]gibberell-16-en-19-oic acid incubation (3 h)

Metabolites (in order of elution)	Disint. min^{-1}	Radioactivity recovered (%)
Gibberellin A_9	7.65×10^5	6·8
Gibberellin A_{15}	5.07×10^5	4·5
Gibberellin A_{24}	1.104×10^6	9·9
Gibberellin A_4	6.36×10^5	5·7
Gibberellin A_7	2.90×10^5	2·6
Gibberellin A_{37}	1.56×10^5	1·4
Gibberellin A_{25}	4.95×10^5	4·4
Gibberellin A_{14}	1.288×10^6	11·5
' Unknowns '	9.42×10^5	8·4
Gibberellin A_{13}	1.95×10^5	1·73
Gibberellin A_3	4.41×10^5	3·95

TABLE 4

ent-[17-^{14}C]Gibberell-16-ene-7,19-dioic acid incubations

Metabolites (in order of elution)	6 h Disint. min^{-1}	6 h Radioactivity recovered (%)	24 h Disint. min^{-1}	24 h Radioactivity recovered (%)
Gibberellin A_9	1.855×10^6	23·3	1.976×10^6	20·9
Gibberellin A_{15}	7.52×10^5	9·4	9.57×10^5	10·1
Gibberellin A_{24}	2.024×10^6	25·4	2.38×10^6	25·2
Gibberellin A_{25}	1.413×10^6	17·7	9.9×10^5	10·5

slowly further metabolised, under the present conditions 89% was recovered unchanged after 144 h further incubation (*cf.* ref. 9). These results suggested a biosynthetic method of preparing the gibberellins A_{24} and A_{25}.[10] Gibberellin A_{12} was prepared synthetically from 7β-hydroxykaurenolide by ring contraction of the 7α-*p*-bromobenzenesulphonate[3] and oxidation of the resultant aldehyde. Incubation of the gibberellin A_{12} with *G. fujikuroi* gave gibberellins A_{24} and A_{25} in 17 and 8% yields.

The experiments with *ent*-7-hydroxygibberell-16-en-19-oic acid (2) on the other hand gave a metabolite distribution which paralleled that of mevalonate when fed under the same conditions. It thus affords a representative picture of the post-ring-contraction metabolite production. In particular the proportions of 3-hydroxylated and non-3-hydroxylated gibberellins are comparable—for example the ratio of A_9 to A_3 activity in the 6 h experiment is almost identical with that in the mevalonate experiment. The results suggest that 3-hydroxylation is more efficient than further oxidation at C-7. An interesting feature arises from a comparison of the distribution of label in the C_{19} and C_{20} fractions. The decrease in the label in gibberellins A_4 (8) and A_7 (5) over the 6—24 h period nearly accounts for the increase in the radioactivity in the gibberellic acid (6). Indeed gibberellin A_4 was converted into gibberellic acid in 30% yield whilst gibberellin A_7 gave gibberellic acid in 43·5% yield. On the other hand, relative to the turnover in the C_{19} fraction, a significant portion of the label remained in the C_{20} fraction. The retention of label in the C_{20} gibberellins was also observed in the non-3-hydroxylated series. It has been shown previously that gibberellin A_{13} was not incorporated into gibberellic acid.[4,11] In our hands gibberellin A_{14} (7) was substantially unchanged after 24 h incubation. MacMillan also reported[6] that gibberellins A_{36} (15) and A_{37} (13) were not metabolised. Whilst it is possible that the δ-lactone and lactol represent blocked functions, another explanation must be sought for gibberellin A_{14}. There is evidence[12] that an earlier precursor, *ent*-kaurene, exists in both free and a bound form linked to a carrier protein. The same might apply in this case and thus affect the transport of the substrate. An alternative possibility is that C-7 remains at the aldehyde oxidation level whilst modification of C-20 takes place.

The results obtained from the incubation of *ent*-7-oxogibberell-16-en-19-oic acid (3) reveal an increase in the proportion of the non-3-hydroxylated gibberellins in comparison with the mevalonate feed—a consequence of the autoxidation of the substrate to gibberellin A_{12}. However the aldehyde still gives a substantial proportion of 3-hydroxylated gibberellins, indicating that it represents the point of divergence between the two series.

EXPERIMENTAL

General experimental details have been described before.[2] Unless otherwise stated, three-day-old cultures (250 ml) of *G. fujikuroi* were used. At this stage the fermentation was not pigmented. The strain of *G. fujikuroi* ACC 917 used in all these experiments was provided by Dr. D. Broadbent (I.C.I. Pharmaceuticals Division, Alderley Park). The fermentations were harvested by filtration; the pH of the broth was then adjusted to pH 2 and the metabolites were recovered by extraction with ethyl acetate. For column chromatography Sephadex LH-20 (165 × 2 cm) was used with the biphasic system light petroleum–ethyl acetate–acetic acid–methanol–water (100 : 80 : 5 : 40 : 7); 250 × 8 ml fractions were collected and 100 μl samples of each fraction were counted. The gibberellins were further purified by t.l.c. on silica (Merck; 0·25 mm thick, 10 × 20 cm) plates. The following solvent systems were used: benzene–acetic acid (9 : 1) for gibberellins A_9, A_{14}, A_{15}, A_{24}, A_{12}, A_{12} alcohol, and A_{12} aldehyde; di-isopropyl ether–acetic acid (95 : 5) for gibberellins A_4, A_7, A_9, A_{13}, A_{14}, A_{24}, and A_{25}; chloroform : ethyl acetate : acetic acid (5 : 5 : 1 and 15 : 5 : 1) for gibberellin A_3; ethyl acetate for gibberellins A_{24} and A_{25}; ethyl acetate–light petroleum (2 : 3) for gibberellins A_{24} and A_{25} as their methyl esters; benzene–ethyl acetate (9 : 1) for gibberellins A_{24} and A_{25} as their methyl esters; benzene : ethanol : ethyl acetate (8 : 1 : 1) for gibberellin A_{37}. G.l.c. was carried out on the methyl esters with a 1% OV 17 column at 230° [nitrogen (50 ml min^{-1}) as carrier gas].

Incubations—(i) [2-^{14}C]*Mevalonic acid*. The mevalonate (25 μCi; 3*R*-isomer; 18 μCi μmol^{-1}) in ethanol (1 ml) was added to one three-day-old culture of *G. fujikuroi*. The fermentation was harvested after 5 days further incubation. The recovery of radioactivity was 21·37 × 10^6 disint. min^{-1} (38·5%) and the product was chromatographed as above.

(ii) ent-7-*Hydroxy*[17-^{14}C]*gibberell-16-en-19-oic acid*. The [17-^{14}C]-alcohol (27·8 × 10^6 disint. min^{-1}; 179 μg) in ethanol (0·2 ml) was added to each of two flasks of *G. fujikuroi*. After 6 h incubation the recovery of radioactivity was 21·49 × 10^6 (77·3%) and after 24 h incubation 23·88 × 10^6 disint. min^{-1} (85·9%). The metabolites were separated and the results are tabulated.

(iii) ent-7-*Oxo*[17-^{14}C]*gibberell-16-en-19-oic acid*. The [17-^{14}C]-aldehyde (14·13 × 10^6 disint. min^{-1}; 92 μg) in ethanol (1·5 ml) was added to one three-day-old culture. After 3 h the fermentation was harvested. The recovery of radioactivity was 11·21 × 10^6 disint. min^{-1} (79·3%). The metabolites were separated and the results are tabulated.

(iv) ent-[17-^{14}C]*Gibberell-16-ene-7,19-dioic acid* (*gibberellin* A_{12}). The [17-^{14}C]-acid (10·92 × 10^6 disint. min^{-1}; 326 μg) in ethanol (0·5 ml) was added to each of two flasks of *G. fujikuroi*. After 6 h incubation the recovery of radioactivity was 7·964 × 10^6 (72·93%) and after 24 h 9·46 × 10^6 disint. min^{-1} (86·62%).

(v) [17-^{14}C]*Gibberellin* A_9. The [17-^{14}C]-acid (3·86 × 10^6 disint. min^{-1}; 84·5 mg) in ethanol (2 ml) was distributed between two two-day-old flasks of *G. fujikuroi*. The fermentation was harvested after a further six days

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incubation. The recovery of gibberellin A_9 was $3{\cdot}40 \times 10^6$ disint. min^{-1} (88·7%) (82 mg). Examination of the crude fermentation extract by radio-t.l.c. failed to reveal any major transformation products.

(vi) *Gibberellin A_{12}*. The acid (60 mg) in ethanol (5 ml) was distributed amongst ten flasks of *G. fujikuroi*. The incubation was continued for 18 h. The pH of the broth was adjusted to pH 2; extraction with ethyl acetate then afforded crude material (240 mg) which was chromatographed on silica gel preparative plates. Further purification (of the methyl esters) by preparative layer chromatography afforded gibberellin A_{25} methyl ester (5 mg) and gibberellin A_{24} methyl ester (11 mg), which were identified by their mass spectra.

(vii) [17-^{14}C]*Gibberellin A_{14}*. The acid ($2{\cdot}297 \times 10^6$ disint. min^{-1}; 491 mg) in ethanol (8 ml) was distributed amongst eight flasks of *G. fujikuroi*. After 24 h further incubation, the fermentation was harvested. The recovery of radioactivity was $2{\cdot}207 \times 10^6$ disint. min^{-1}. A sample was methylated with diazomethane and analysed by g.l.c. The major product has a retention time of 6·1 min, identical with that of gibberellin A_{14} methyl ester. Examination of the fermentation extract by radio-t.l.c. failed to reveal any major transformation products.

(viii) [17-^{14}C]*Gibberellin A_4*. The acid ($1{\cdot}525 \times 10^6$ disint, min^{-1}) in ethanol (1 ml) was aded to one three-day-old flask of *G. fujikuroi*. The incubation was continued for a further 24 h and then the metabolites were harvested (total recovery $1{\cdot}102 \times 10^6$ disint. min^{-1}). The gibberellic acid ($4{\cdot}63 \times 10^5$ disint. min^{-1}; 30·3% incorporation) was separated by chromatography on Sephadex LH 20 (36 × 4 cm) in the biphasic system already described and its purity was confirmed by radio-t.l.c.

(ix) [17-^{14}]*Gibberellin A_7*. The acid ($3{\cdot}76 \times 10^5$ disint. min^{-1}) in ethanol (1 ml) was added to one three-day-old flask of *G. fujikuroi*. The incubation was continued for a further 24 h and then the metabolites (total recovery $3{\cdot}289 \times 10^5$ disint, min^{-1}) were isolated. The gibberellic acid ($1{\cdot}63 \times 10^5$ disint. min^{-1}; 43·5% incorporation) was separated and purified as above.

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