Fungal Products. Part XIV.[1] Metabolic Pathways from *ent*-Kaurenoic Acid to the Fungal Gibberellins in Mutant B1-41a of *Gibberella fujikuroi*

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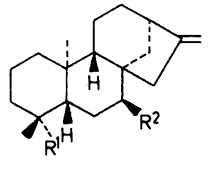
The metabolism of substrates normally produced by wild-type strains of *Gibberella fujikuroi* has been determined in resuspended cultures of the mutant B1-41a at pH 3·5 and 7·0. The metabolites were identified by g.l.c.–mass spectrometry and g.l.c–radio-counting. From the results at pH 3·5 the metabolic steps from *ent*-kaurenoic acid to the fungal gibberellins have been deduced. The oxidation level at which C-20 is lost in the formation of the C_{19}-gibberellins could not be determined. *ent*-7-Oxokaurenoic acid does not serve as a precursor of gibberellin A_{12} aldehyde. At pH 7·0 the pathway from gibberellin A_{12} is diverted completely to 3-deoxygibberellins such as gibberellin A_9.

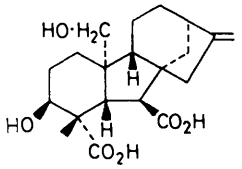
In Part XI it was shown [2] that the mutant B1-41a, derived from the wild type strain GF-1a of *Gibberella fujikuroi*, was blocked for diterpenoid biosynthesis at the step between *ent*-kaur-16-en-19-al (1) and *ent*-kaur-16-en-oic acid (2). The leakage at the block was less than 3% so that metabolites produced by wild-type strains from *ent*-kaurenoic acid (2) are present in very low concentrations in cultures of the mutant B1-41a, particularly when resuspended cultures are used. This mutant strain therefore provides an excellent system for the detailed study of diterpenoid biosynthesis in *G. fujikuroi*. In the near absence of metabolites derived from *ent*-kaurenoic acid (2), the metabolism of added substrates can be followed without using radioactive labels. A further advantage of the system is that the added substrates do not compete with endogenously produced metabolites for enzyme sites. These advantages, combined with the use of g.l.c.–mass spectrometry for rapid, semiquantitative analysis of metabolites, have enabled us to study the metabolic pathways from *ent*-kaurenoic acid (2) to the fungal gibberellins (GAs).

The approach [3,4] comprised a study of the metabolism of substrates which are known metabolites of wild-type strains of *G. fujikuroi* and which either had been shown to act as precursors of fungal GAs by previous tracer studies in wild-type strains or possessed the structures of possible intermediates in the GA biosynthetic pathway. Resuspended cultures [2] of the mycelium of the mutant were used to ensure reproducibility, to remove endogenous metabolites from the original culture medium, and to facilitate changes, such as pH, in the conditions of culture. The results of experiments conducted at pH 3·5 are shown in the Table and interpreted in the Scheme.

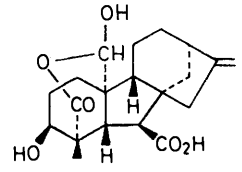
The formation of GA_{12} aldehyde from *ent*-7α-hydroxykaurenoic acid (3) [5] and its conversion into GA_{14} aldehyde [6] have been demonstrated previously by radio-labelling techniques. The occurrence of all other steps shown in the Scheme was established by feeding the precursor for that step and identifying its immediate metabolite by g.l.c.–mass spectrometry and also, in



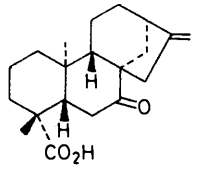
(1) R^1 = CHO, R^2 = H
(2) R^1 = CO_2H, R^2 = H
(3) R^1 = CO_2H, R^2 = OH



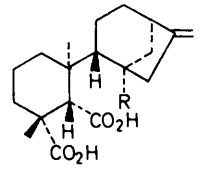
(4)



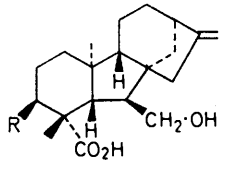
(5)



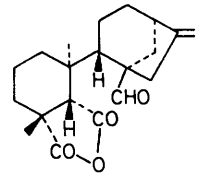
(6)



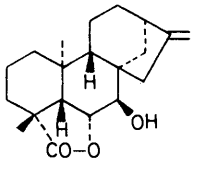
(7) R = CO_2H
(8) R = CHO



(9) R = H
(10) R = OH



(11)



(12)

selected cases, by g.l.c.–radio-counting.[7] Although some of the individual steps, discussed later, have been

[1] Part XIII, R. C. Durley, T. J. Simpson, J. MacMillan, A. T. Glen, and W. B. Turner, *J.C.S. Perkin I*, 1975, 163.
[2] J. R. Bearder, J. MacMillan, M. B. Chaffey, and B. O. Phinney, *Phytochemistry*, 1974, **13**, 911.
[3] Preliminary report, J. R. Bearder, P. Hedden, J. MacMillan, C. M. Wels, and B. O. Phinney, *J.C.S. Chem. Comm.*, 1973, 777.
[4] Preliminary report, J. R. Bearder, V. R. Frydman, P. Gaskin, W. E. Harvey, P. Hedden, J. MacMillan, B. P. Phinney, and C. M. Wels, in 'Plant Growth Substances 1973,' ed. S. Tamura, Hirokawa, Tokyo, in the press.
[5] J. R. Hanson and A. F. White, *J. Chem. Soc.* (*C*), 1969, 981.
[6] P. Hedden, J. MacMillan, and B. O. Phinney, *J.C.S. Perkin I*, 1974, 587.
[7] J. MacMillan and C. M. Wels, *Phytochemistry*, 1974, **13**, 1413.

established previously by other investigators the pathways shown in the Scheme are derived from an integrated study using a single set of conditions.

In discussing the results presented in the Table and Scheme it is convenient to work backwards from GA_3, which is the major terminal gibberellin in wild-type strains and which has been found in the present study not to be metabolised by the mutant B1-41a. Gibberellin A_4 was almost completely metabolised to GA_3 but small quantities of GA_7, GA_{16}, and GA_1 were formed. Gibberellins A_1 and A_{16} were not metabolised when re-fed and are thus terminal gibberellins. Gibberellin A_{16} is not therefore a precursor of GA_7 or a product of GA_7 (*cf.* ref. 8). These results agree with those of Pitel *et al.*,[9] who have established the same pathway from GA_4 in the wild-type strain ACC 917, and with our previous observation [10] that GA_1 is only converted into GA_3 in very low yield in the mutant R-9 of *G. fujikuroi*. Although GA_{14} was metabolised slowly (a point which is discussed later) it was converted into the known 3-hydroxylated fungal gibberellins A_4, A_7, A_3, A_1, A_{16}, A_{13}, A_{36}, and A_{42} (16,17-dihydro-16-hydroxy-GA_{14}). Gibberellins A_2 and A_{37}, the only other known 3-hydroxylated GAs, were not detected. Gibberellin A_{42}, formed from GA_{14} within 20 h, remained unmetabolised when re-fed.

In an attempt to determine the intermediate (2) between GA_{14} and GA_4, the potential candidates GA_{37}, GA_{36}, and GA_{13}, which represent progressive oxidation of the carbon atom lost in this conversion, were re-fed under conditions in which GA_{14} was metabolised. Gibberellin A_{13} anhydride was included in this series of experiments since Hanson and Hawker [11] have recently observed a low conversion of the ^{14}C-labelled anhydride into GA_3. Even after 5 days incubation GA_{37}, GA_{36}, and GA_{13} were not metabolised and GA_{13} anhydride was completely hydrolysed to GA_{13}. Even in 20 h feeds in which high concentrations of the anhydride were used so that some remained unhydrolysed, no metabolites of GA_{13} anhydride were detected. The non-metabolism of GA_{37} is explicable if the true intermediate is the corresponding hydroxy-acid (4) which is not equivalent to, or formed from, the exogenously supplied lactone. The latter could then be a terminal gibberellin derived from the hydroxy-acid (4). Some evidence for this possibility has been presented by Graebe *et al.*,[12] as a result of time-course studies with a cell-free system from *Cucurbita maxima*. The non-metabolism of GA_{36}, even to GA_{13}, suggests either lack of penetration or an unfavourable equilibrium between the free aldehyde function and the lactol (5).[13] The possibility that metabolism of GA_{36} required enzyme induction by a precursor was tested by feeding cold GA_{36}

Metabolites of substrates incubated with B1-41a at pH 3·5 for 20 h

Substrate	Metabolites (% total; ±3%) ‡																		
	GA_3	GA_1	GA_{16}	GA_{13}	(11)	(7)	GA_9	GA_{24}	GA_{25}	(12)	GA_{42}	GA_4	GA_7	(8)	GA_{14}	GA_{15}	GA_{12} anh.	GA_{25}	GA_{36}	GA_{37}
ent-Kaurenoic acid (2)	28	2	1	7	6	4	1	1	1	2	1	4	1	21	22					
ent-7α-Hydroxy-kaurenoic acid (3)	41	1		4		4	3	1		8				28	10					
ent-7-Oxokaurenoic acid (6)						100														
GA_{12} alcohol (8) *	17			5								2	2		73					
GA_{12} alcohol (8)	28			6								2	2		62					
GA_{12} aldehyde *	4						4		1			1			85		3			
GA_{12} aldehyde	20	2	1	4			6	2	2			2	3		54	2	2			
GA_{12} aldehyde †	52	1	1	9			9	1	10		7	2	1		5	2				
GA_{12} *							37	7	11								40			
GA_{12}	7			2			58	14	4						8	5	2			
GA_{14} aldehyde	34			8								2	2		54					
GA_{14}	32	2	1	4							1	4	4		50				1	
GA_{24}								45	51									4		
GA_{25} †									100											
GA_{15} †																100				
GA_{36} †																			100	
GA_{37} †																				100
GA_4	79	2	2									2	15							
GA_4 *												100								
GA_7	71												29							
GA_7 *													100							
GA_{16}			100																	
GA_1		100																		
GA_3 *	100																			
GA_{42}											100									
GA_{13} anhydride				100																

* At pH 7·0. † For 5 days. ‡ Determined by g.l.c. on 2% QF1 as methyl esters or methyl ester trimethylsilyl ethers.

[8] M. Katsumi and B. P. Phinney in ' The Gibberellins,' ed. S. Tamura, Tokyo University Press, 1968, ch. 4.

[9] D. W. Pitel, L. C. Vining, and G. P. Arsenault, *Canad. J. Biochem.*, 1971, **49**, 194; A. G. McInnes, D. G. Smith, G. P. Arsenault, and L. C. Vining, *ibid.*, 1973, **51**, 1470.

[10] J. R. Bearder, J. MacMillan, and B. O. Phinney, *Phytochemistry*, 1973, **12**, 2655.

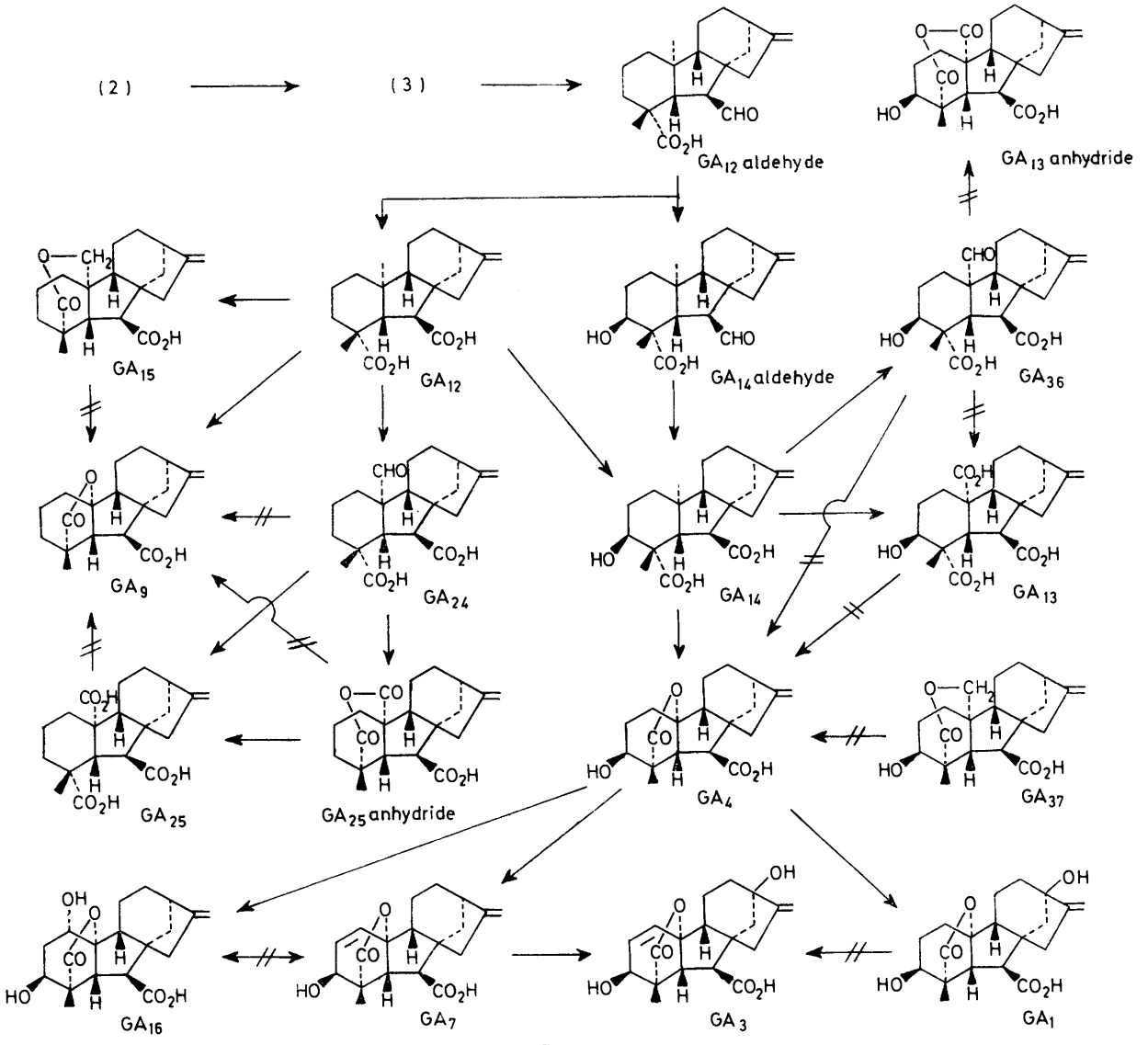
[11] J. R. Hanson and J. Hawker, *Tetrahedron Letters*, 1972, 4299.

[12] J. E. Graebe, P. Hedden, and J. MacMillan, in ' Plant Growth Substances, 1973,' ed. S. Tamura, Hirokawa, Tokyo, in the press.

[13] J. R. Bearder and J. MacMillan, *J.C.S. Perkin I*, 1973, 2824.

with [6-^{3}H]GA_{12} aldehyde. G.l.c.–radio-counting of the products, however, showed that GA_{14} and GA_3 had approximately the same specific activity; thus GA_3 had not been produced *via* cold GA_{36}. The non-metabolism of GA_{13} to GA_3 in the wild-type strain ACC 917 has been shown previously by Cross *et al.*[14]

GA_{12} aldehyde to the 3-hydroxylated GAs. When [6-^{3}H]GA_{12} was fed for 20 h and the methylated metabolites were examined by g.l.c.–radio-counting [Figure 1(B)] 90% of the radioactivity was recovered. Two-thirds of the radioactivity was present in the 3-deoxy-GAs, GA_{15}, GA_{24}, GA_{25}, and GA_9. This result agrees



SCHEME

Gibberellin A_{14} aldehyde was rapidly metabolised to GA_{14}; after 20 h the product distribution was identical with that of a 20 h feed of GA_{14}. Similarly GA_{12} aldehyde was rapidly metabolised and, after 20 h, gave all the 3-hydroxylated GAs obtained from a similar feed of GA_{14}. However the 3-deoxy-GAs, GA_{15}, GA_{24}, GA_{25}, and GA_9 were also produced. A radio-g.l.c. trace of the products after a 20 h incubation of [6-^{3}H]GA_{12} aldehyde is shown in Figure 1(A). A g.l.c. trace of a 5-day feed is shown in Figure 2; all identifications were confirmed by g.l.c.–mass spectrometry.

Gibberellin A_{12} is not on the main pathway from

with that of Cross *et al.*,[14] who found that [^{14}C]GA_{12} was incorporated into [^{14}C]GA_3 in lower yield than [^{14}C]GA_{12} aldehyde. However these authors did not identify the other products from [^{14}C]GA_{12}. The present results thus show for the first time that GA_{12} is an efficient precursor of the 3-deoxy-GAs. The effect of higher pH on the metabolism is discussed later.

As in the 3-hydroxylated pathway the lactone (GA_{15}), the aldehyde (GA_{24}), the acid (GA_{25}), and GA_{25} anhydride did not act as precursors of GA_9 when fed to

[14] B. E. Cross, K. Norton, and J. C. Stewart, *J. Chem. Soc.* (*C*), 1968, 1054.

cultures of the mutant. Gibberellins A_{15} and A_{25} were not metabolised but GA_{24}, in contrast to GA_{36}, was converted into the corresponding acid and the anhydride. Gibberellin A_{25} anhydride was hydrolysed to the free acid, GA_{25}. Thus our results do not define the oxidation state at which C-20 is lost in the conversion of C_{20} GAs into C_{19} GAs. Our negative results may be due to the inability of the added substrates to penetrate cell membranes, one of the limitations of metabolic studies with intact organisms (for a review see Brown [15]). Gibberellin A_{13} anhydride may indeed be the precursor to GA_4, as indicated by the work of Hanson and Hawker,[11] and it is interesting that we have observed more rapid hydrolysis of GA_{13} and GA_{25} anhydrides in the mutant cultures (80% in 20 h) than in controls (10% in 20 h).

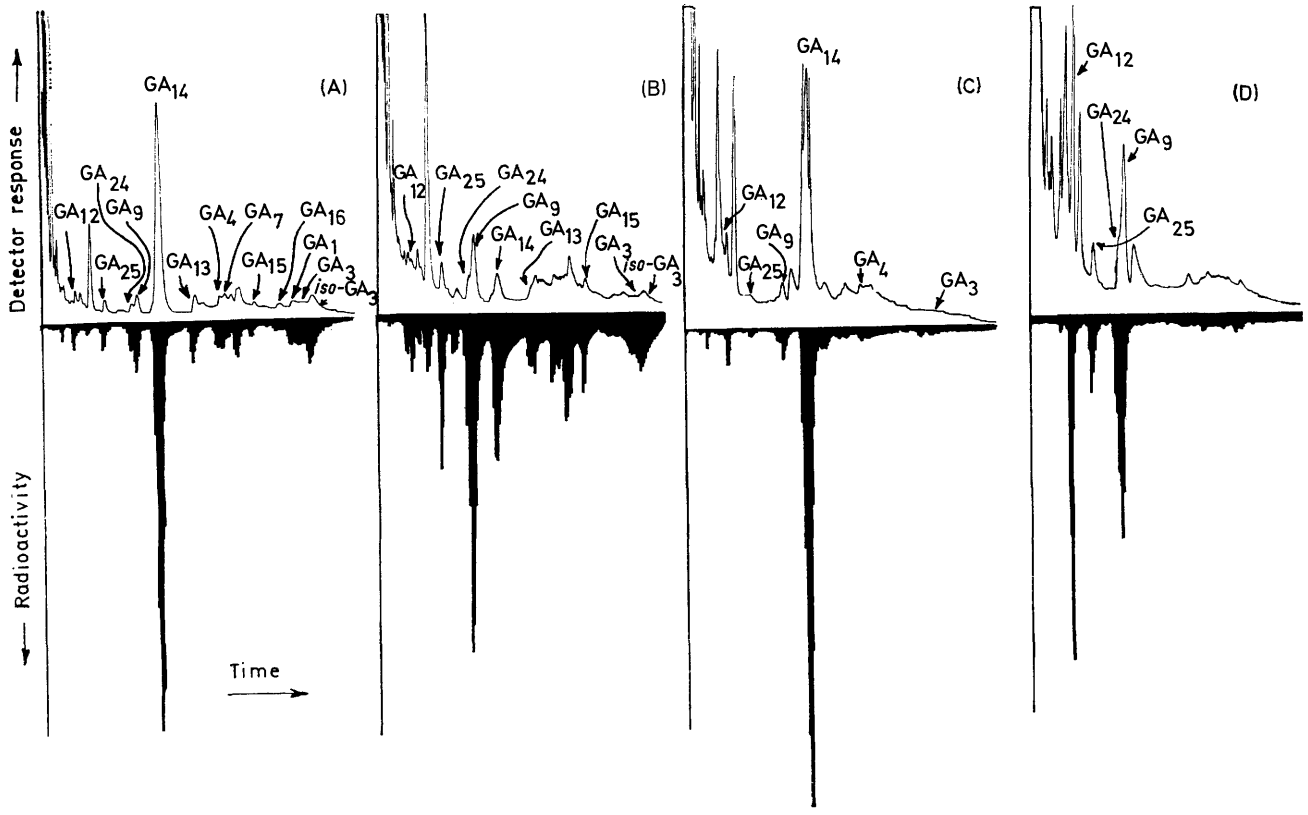


FIGURE 1 G.l.c.–radio-counting of metabolites from [6-^{3}H]GA_{12} aldehyde at (A) pH 3·5 and (C) pH 7·0 and from [6-^{3}H]GA_{12} at (B) pH 3·5 and (D) pH 7·0; g.l.c. conditions: 3% QF-1 at 170° for 35 min then 170—205° at 2° min^{-1}; metabolites identified by g.l.c.–mass spectrometry

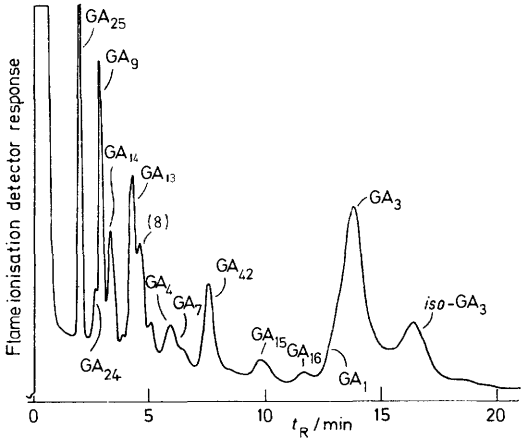


FIGURE 2 G.l.c. trace of total methylated acidic extract from a GA_{12} aldehyde 5 day feed to *G. fujikuroi*, mutant B1-41a at pH 3·5 (2% QF-1 column at 210°); identification by g.l.c.–mass spectrometry

ent-7α-Hydroxykaurenoic acid (3) and *ent*-kaurenoic acid (2) [2] were metabolised in a similar manner by the mutant cultures, giving the metabolites shown in the Table. The total ion current traces (t.i.c.) obtained by g.l.c.–mass spectrometry of the derivatised extracts from the culture medium of feeds of each compound were qualitatively identical with that obtained from wild-type cultures of the parent strain GF-1a except for the concentration of GA_{14}. The latter was much lower in the wild-type cultures and this fact is discussed later. Although equal concentrations of *ent*-kaurenoic acid (2) and *ent*-7α-hydroxykaurenoic acid (3) were fed to the same amount of resuspended mycelium of the mutant the absolute concentration of the metabolites from *ent*-7α-hydroxykaurenoic acid was *ca.* ten times less than that from *ent*-kaurenoic acid. Although little or no *ent*-7α-hydroxykaurenoic acid remained in the

[15] S. A. Brown in ' Biosynthesis,' ed. T. A. Geisman, Chem. Soc. Specialist Periodical Report, 1972, vol. 1, p. 1.

culture medium, considerable amounts were extracted from the mycelium with methanol. These observations confirm the results of Lew and West,[16] who found that *ent*-kaurenoic acid was converted into GA_3 by a wild-type strain of *G. fujikuroi* (*ca.* 12% incorporation) but the *ent*-7α-hydroxy-derivative was only incorporated to the extent of *ca.* 4%. The step from *ent*-7α-hydroxy-kaurenoic acid to GA_{12} aldehyde, previously established by Hanson and White [5] was not directly established in our work. The step from *ent*-kaurenoic acid (2) to *ent*-7α-hydroxykaurenoic acid (3), recently reported [17] in a cell-free system from *G. fujikuroi*, was also established in the present study in incubations of less than 1 h. Thus, as shown in the Scheme, *ent*-kaurenoic acid (2) and *ent*-7α-hydroxykaurenoic acid serve as efficient precursors to the C_{19} and C_{20} GAs.

Two compounds, at present not known metabolites of wild-type strains of *G. fujikuroi*, were examined as possible intermediates between *ent*-7α-hydroxykaurenoic acid (3) and GA_{12} aldehyde. First, *ent*-7-oxokaurenoic acid (6) was completely metabolised after 1 day to a single compound which was initially [3] assumed to be the unchanged ketone (6) (g.l.c.); subsequent g.l.c.–mass spectrometry, however, has identified this compound as the *seco*-ring B triacid (7). Secondly, GA_{12} alcohol (9), previously shown [10,18] to be an effective precursor of C_{19} GAs, was predictably metabolised by cultures of the mutant B1-41a. However no 3-deoxy-GAs were detected, suggesting that GA_{12} alcohol (9) was metabolised mainly *via* GA_{14} alcohol (10) rather than GA_{12} aldehyde. The conversion of GA_{14} alcohol (10) into 3-hydroxy-GAs by the mutant B1-41a has been previously described.[6] The status of GA_{12} and GA_{14} alcohols as endogenous intermediates in the GA pathway merits further investigation.

As noted earlier, GA_{14} accumulated in 20 h feeds of *ent*-kaurenoic acid (2), *ent*-7α-hydroxykaurenoic acid (3), GA_{12} aldehyde, and GA_{14} aldehyde. This accumulation was only temporary since GA_{14} was almost completely metabolised after 5 days (see, for example, Figure 2). It appears therefore that the conversion of GA_{14} into GA_4 is the rate-limiting step in the overall pathway from *ent*-kaurenoic acid to GA_3 in the mutant B1-41a. Since *ent*-7α-hydroxykaurenoic acid, GA_{12} aldehyde, and GA_{14} aldehyde were not detected even in the 20 h feeds in which GA_{14} accumulated, it appears that GA_{14} does not control its own formation from *ent*-kaurenoic acid in this mutant. Since GA_{14} does not accumulate to the same extent in the wild-type parent, GF-1a, the rate-limiting step for GA_3 production in this strain probably occurs before *ent*-kaurenoic acid.

Some of the conversions upon which the Scheme is based were also examined in resuspended cultures of the mutant B1-41a, buffered at pH 7. Under these conditions, reported [19] to favour the production of GA_7 and GA_9 over GA_3 in the wild-type strain ACC 917, GA_4 and GA_7 were not metabolised and the metabolism of GA_{14} appeared to be suppressed. Thus [6-^{3}H]GA_{12} aldehyde was metabolised mainly to GA_{14} and gave traces only of GA_4 and GA_3 [see Table and Figure 1(C)]. The effect of the higher pH on the branch from GA_{12} to the 3-hydroxy- and 3-deoxy-GAs was even more marked. At pH 7 two-thirds of the [6-^{3}H]GA_{12} had been metabolised after 20 h almost entirely to GA_9, with traces of GA_{24} and GA_{25} [Figure 1(D)]; GA_{14} and its metabolites were not detected. These differences noted at the two pH values do not necessarily reflect changes in enzyme activity; they may be due entirely to the effect upon substrate transport of greater ionisation at higher pH.

In conclusion it is emphasised that the pathway shown in the Scheme applies strictly to the mutant B1-41a. However, there is strong evidence that essentially the same pathways operate in wild-type strains. Thus the qualitative and (apart from GA_{14}) the quantitative distributions of the metabolites derived from feeds of *ent*-kaurenoic acid (2) to the mutant are very similar to that of the normal metabolites from the wild-type parent GF-1a.[2,7] Also all previous results of other investigators using wild-type strains can be accommodated in the Scheme.

EXPERIMENTAL

For general experimental details see Part V; [20] for g.l.c.–mass spectroscopy see Part XI.[2]

Feeding Experiments with G. fujikuroi, *Mutant B1-41a.*—Resuspension cultures of pigmented mycelium were prepared as described in Part XI.[2] The pH of the resuspension medium was adjusted to 3·5 with 2N-sulphuric acid or to 7 with 2N-potassium hydroxide. The substrate (0·5—1·0 mg) was dissolved in the minimum volume of acetone and added to sterile water (5 ml) in a shake flask (100 ml) immediately after autoclaving. When the flask was cool, mycelial suspension (20 ml) was added and the culture was incubated under the usual conditions.

Feeds of Unlabelled Substrates.—These were worked up as follows. The total culture was centrifuged at 4000 rev. min^{-1} for 20 min and the supernatant fraction was collected. After acidification to pH 2·5 with 2N-hydrochloric acid this fraction was extracted with ethyl acetate (50 ml). Recovery from the extract gave the product mixture which was methylated with diazomethane; a portion of the resulting methyl derivative was trimethylsilylated with Sweeley's reagent.[21]

The mycelium was shaken with methanol (30 ml) at 20° for 24 h. After filtration the methanol was evaporated off under vacuum and the residue was partitioned between ethyl acetate and aqueous acid (pH 2·5). The material recovered from the ethyl acetate was derivatised as above.

In g.l.c. and g.l.c.–mass spectrometry the products from the culture medium were examined as the methyl derivatives on the more polar QF-1 or OV210 columns (conditions A, 210°; ref. 2) although some were also analysed under

[16] F. T. Lew and C. A. West, *Phytochemistry*, 1971, **10**, 2065.
[17] C. A. West in 'Biosynthesis and its Control in Plants,' ed. B. V. Milborrow, Academic Press, London and New York, 1973, p. 143.
[18] J. R. Hanson and J. Hawker, *Phytochemistry*, 1973, **12**, 1073.
[19] B. E. Cross, R. H. B. Galt, and J. R. Hanson, *Tetrahedron*, 1962, **18**, 491.
[20] J. MacMillan and T. J. Simpson, *J.C.S. Perkin I*, 1973, 1487.
[21] C. C. Sweeley, R. Bentley, M. Makita, and M. M. Wells, *J. Amer. Chem. Soc.*, 1963, **85**, 2497.

conditions B[2] at 210°. The methyl trimethylsilyl derivatives were analysed under either set of conditions. All mycelial extracts were analysed under conditions B[2] at 200°.

Feeds of Labelled Substrates.—The following were fed to resuspended cultures (20 ml) at both pH 3·5 and 7·0 for 20 h: (*a*) [6-^{3}H]GA_{12} aldehyde ($2·77 \times 10^6$ disint. min^{-1}); (*b*) [6-^{3}H]GA_{12} alcohol ($10·3 \times 10^6$ disint. min^{-1}); (*c*) GA_{12} ($11·7 \times 10^5$ disint. min^{-1}); and (*d*) [6-^{3}H]GA_{12} aldehyde ($2·77 \times 10^6$ disint. min^{-1}) together with inactive GA_{36} (*ca.* 500 μg) at pH 3·5 only. These feeds were worked up by filtration (not by centrifugation). The filtrates were acidified to pH 2·5 and extracted with ethyl acetate (3 × 100 ml). The radioactivity recovery in all cases was 94—99·5%.

Each extract was methylated and analysed by g.l.c.–radio-counting as previously described [7] under the following conditions: hydrogen pressure 17 lb in^{-2}; air pressure 15 lb in^{-2}; nitrogen flow rate 75 ml min^{-1}; total gas flow *ca.* 400 ml min^{-1}. The results for the GA_{12} and GA_{12} aldehyde feeds are shown in Figure 1. The g.l.c.–radio-counting trace of the feed of GA_{12} aldehyde in the presence of GA_{36} was almost identical to that of the GA_{12} aldehyde feed at pH 3·5 except for the higher mass peak in the flame ionisation detector trace in the former case.

The compounds shown in Figure 1 were identified by g.l.c.–mass spectrometry under g.l.c. conditions C,[2] with temperature programmed from 180 to 230° at 2° min^{-1}.

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