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STUDIES ON THE BIOSYNTHESIS OF GIBBERELLINS FROM (−)-KAURENOIC ACID IN CULTURES OF *GIBBERELLA FUJIKUROI*

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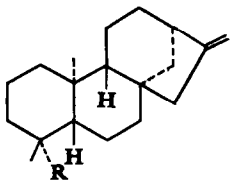
Abstract—*Gibberella fujikuroi* utilizes (−)-kaur-16-en-19-oic acid as a precursor for the synthesis of gibberellins. A study of the time-course of the conversion of kaurenoic acid into gibberellins provides evidence that the sequence of steps involves an overall change toward higher oxidation levels, with GA-4 and GA-7 at an earlier stage on the synthetic pathway than GA-1 and GA-3.

INTRODUCTION

THE observation by Katsumi *et al.*[1,2] that (−)-kaur-16-en-19-oic acid (I)[3] and some related diterpenoid compounds stimulated seedling elongation in the dwarf maize mutants d-5 and an-1 has led to the investigation of the role of (I), the corresponding kaurenol (II), and (−)-kaurene (III) in gibberellin biosynthesis. The gibberellin-like activity of the kaurene derivatives in dwarf maize suggested the possibility that they were converted in these mutants into gibberellins and that the growth response was a reflection of the biosynthetic pathways by which the natural gibberellins are formed.



(I) R = COOH
(II) R = CH_2OH
(III) R = CH_3

An early suggestion that gibberellin A-3 (GA-3) is produced in *Gibberella fujikuroi* cultures from diterpenoid intermediates was first made by Birch *et al.* who carried out feeding

* Contribution No. 1960 from Department of Chemistry, UCLA.

[1] M. KATSUMI, B. O. PHINNEY, P. R. JEFFERIES and C. A. HENRICK, *Science* **144**, 849 (1964).
[2] B. O. PHINNEY, P. R. JEFFERIES, M. KATSUMI and C. A. HENRICK, *Plant Physiol. Suppl.* **39**, XXVII (1964).
[3] C. A. HENRICK and P. R. JEFFERIES, *Chem. & Ind. (London)* 1801, 1802 (1963).

experiments with carbon-14-labeled acetic and mevalonic acids which indicated that gibberellic acid was formed through the normal isoprenoid pathway of terpene biosynthesis.[4] The general scheme of synthesis of compounds of this class has been discussed by Wenkert.[5]

Further support for this view was recently provided in a study by Cross, Galt and Hanson,[6] the results of which appeared while the present work was in progress. They found that (−)-kaurene-17-^{14}C was transformed by *G. fujikuroi* cultures into gibberellic acid (GA-3) without alteration of the position of the label. The gibberellin GA-9, similarly labeled was, however, not converted to GA-3 when fed to the fungus culture. The discovery of (−)-kaurene itself in natural cultures of the fungus[7] adds further support to the view that the pathway of terpenoid synthesis through kaurene is the process by which these growth-promoting compounds arise in nature. The operation of this pathway in higher plants has recently been observed. In homogenates of endosperm-nucellus tissue of immature *Echinocystis macrocarpa* (wild cucumber) seed, (−)-kaurene and (−)-kauren-19-ol were produced from mevalonic acid-2-^{14}C.[8]

While these studies have provided evidence in support of a general pathway of gibberellin synthesis by the isoprenoid route, little evidence of a comparable kind exists concerning the metabolic relationships between the numerous gibberellins themselves. The recent isolation[9] of a number of new kaurene-related metabolites from *Gibberella fujikuroi* provides material for much speculation in this regard but definitive experiments are still lacking.

The gibberellin-like activity of kaurenol (II) and kaurenoic acid (I) and the accessibility of these compounds in labeled (at C_{17}) form led to the work described in this paper. Our primary purpose was to use the fungus as an aid in parallel studies of the fate of kaurenol and kaurenoic acid in maize mutants, and advantage was taken of the opportunity to examine the metabolic products formed in the fungus cultures.

RESULTS

Synthetic Growth Medium for Culture of G. fujikuroi

Borrow *et al.*[10–12] noted that most of the final yield of gibberellins is produced by the fungus after the maximum dry weight of mycelium has been attained and the rapid uptake of sugar and ammonia nitrogen has ceased. Also, Cross and his co-workers, in their study of the metabolism of (−)-kaurene by the fungus,[6] found it expedient to add the hydrocarbon to the culture when the nitrogen of the solution had become exhausted. With these observations in mind, three media were prepared with varying amounts of nitrogen as the limiting nutrient.

1. PDL, Potato dextrose liquor, a potato starch extract of unknown nitrogen content.[13]

[4] A. J. BIRCH, R. W. RICKARDS and H. SMITH, *Proc. Chem. Soc.* 192 (1958). A. J. BIRCH, R. W. RICKARDS, H. SMITH, A. HARRIS and W. B. WHALEY, *Tetrahedron* **7**, 241 (1959).

[5] E. WENKERT, *Chem. & Ind. (London)* 282 (1955).

[6] B. E. CROSS, R. H. B. GALT and J. R. HANSON, *J. Chem. Soc.* 295 (1964).

[7] B. E. CROSS, R. H. B. GALT, J. R. HANSON, P. J. CURTIS, J. F. GROVE and A. MORRISON, *J. Chem. Soc.* 2937 (1963).

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[10] A. BORROW, E. G. JEFFERYS, R. H. J. KESSELL, E. C. LLOYD, P. B. LLOYD and J. S. NIXON, *Can. J. Microbiol.* **7**, 227 (1961).

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[13] K. C. JONES, Isolation and Characterization of a New Gibberellin Produced by *Fusarium moniliforme* (Saw.) Wr. Ph.D. Thesis submitted to the University of California, Los Angeles (1964).

2. ICI, The medium used by the Akers Laboratory group with 4·8 g/l., or 10 times the amount of ammonium nitrate, was found to be unsatisfactory for gibberellin production under our conditions of growth. 3. 10% ICI, The normal ICI medium with 10% of the amount of ammonium nitrate as follows: 80 g, glucose; 0·48 g, NH_4NO_3; 5·0 g, KH_2PO_4; 1·0 g, $MgSO_4{:}7H_2I$; 2 ml of the following trace element solution: 0·1 g, $FeSO_4 \cdot 7H_2O$; 0·015 g, $CuSO_4 \cdot 5H_2O$; 0·161 g ,$ZnSO_4 \cdot 7H_2O$; 0·01 g, $MnSO_4 \cdot 7H_2O$; 0·01 g, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (ammonium molybdate); distilled water to 100 ml; the complete medium being made up to 1 l. with distilled water.

The media were inoculated with *G. fujikuroi* Lilly strain M-119, incubated at 26° in De Long culture flasks on a rotary shaker, and the gibberellins produced were isolated. Total gibberellin production was estimated by means of assay on dwarf d-5 mutant maize seedlings, and by observation of the intensity and variety of the blue fluorescing zones on thin-layer chromatograms. The 10% ICI culture was found to produce gibberellins in both the quantity and variety desirable for our purposes in a reasonably short time. Although the potato dextrose medium also gives a good variety and quantity of gibberellins in a minimum of time, the 10% ICI medium was chosen for use because it is a synthetic medium of known composition. Furthermore, the low nitrogen media turned pink on the third day of incubation, and this color change seems to correlate approximately with an increased rate of gibberellin production. The procedure adopted as a result of this study was to use the appearance of the pink color as an indication of the best time to add the radioctive compound being studied.

Biosynthesis Studies

A culture of *Gibberella fujikuroi* was grown for either 3 or 7 days in the 10% ICI medium, at which time (−)-17-^{14}C-kaurenoic acid was fed. The mycelium was harvested after periods of growth described below. Conversion to metabolic products in the 4-hr run was high (Table 1) and recovery of purified "acidics" (Fraction E, see Experimental section) compared

TABLE 1. SUMMARY OF FEEDING EXPERIMENTS

Feeding time	4 hours		2 days		7 days		15 days		35 days	
Days growth before feeding	7		3		3		3		3	
Sample	1	2	1	2	1	2	1	2	1	2
^{14}C-kaurenoic acid*	5·0	5·0	5·5	5·5	6·9	6·9	6·9	6·9	6·9	6·9
Mother liquors (A)	0·19	0·10	0·32	0·27	0·34	0·36	0·59	0·66	0·90	0·89
Neutrals (B)	0·36	0·34	0·40	0·30	0·43	0·44	0·56	0·51	0·53	0·53
Aqueous misc. (C)	0·04	0·04	0·14	0·11	0·16	0·17	0·34	0·42	0·23	0·19
Acidics crude (D)	2·61	2·48	2·77	2·68	4·10	4·10	4·68	4·68	4·42	4·40
Total recovery	3·20	2·96	3·63	3·36	5·03	5·07	6·17	6·30	6·08	6·01
Recovery (%)	64	59	66	61	73	73	90	91	88	87
Acidics purified (E)	2·06	2·28	2·09	2·37	2·77	2·78	3·74	3·70	3·54	3·83
(E) as % of feed	41	46	38	43	40	40	54	54	52	56

* Kaurenoic acid fed in μc. Figures for recoveries are in μc.

well with the results of longer feeding periods: 41 and 46 per cent of the administered radioactivity was found in this fraction in the two 4-hr experiments. This activity is due to acidic conversion products since it has been found, and confirmed by control experiments, that under the conditions used in the separation procedure kaurenoic acid partitions into the

neutral phase. This was established by the observations that (*a*) no radioactivity was found at the R_f value for kaurenoic acid on TLC plates of the acidic E fractions; (*b*) TLC in solvent system 4 (see Experimental) of the "neutral" fraction B from a 7-day experiment showed a radioactive spot corresponding in position and blue u.v.-fluorescence to authentic kaurenoic

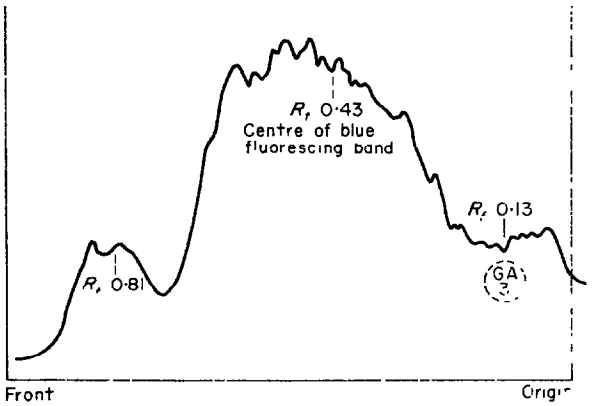


FIG. 1. PREPARATIVE TLC OF THE ACIDIC FRACTION OF *Gibberella fujikuroi* AFTER FEEDING WITH (−)-17-^{14}C-KAURENOIC ACID.

Scan 46: 4-hour feed after 7-day growth; purified acidics; charge, 2·06 μc; range, 30,000. Authentic standard: GA-3, R_f 0·13. Analysis: R_f 0·13; a strongly blue-fluorescing band and radio-peak distinctly appear in rechromatography of this zone. GA-1 identified by dilution and recrystallization to constant specific activity. Also contains GA-3. R_f 0·43; a broad blue-fluorescing radio-peak resolved on rechromatography into GA-4, GA-7 and compound B (see text). All contributed to bio- and radio-activity. R_f 0·81: Blue-fluorescing, radioactive, bioactive. Probably GA-9.

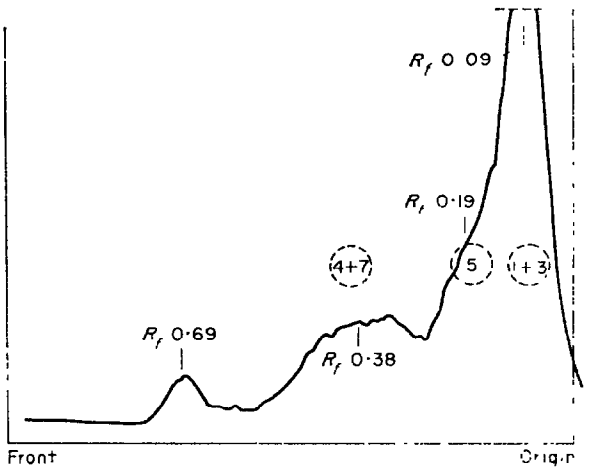


FIG. 2. PREPARATIVE TLC OF THE ACIDIC FRACTION OF *Gibberella fujikuroi* AFTER FEEDING WITH (−)-17-^{14}C-KAURENOIC ACID.

Scan 37: 2-day feed after 3-day growth; purified acidics; charge, 2·09 μc; range, 30,000. Authentic standards: GA-3, R_f 0·11; GA-14+7, R_f 0·40. Analysis: R_f 0·09; a strongly blue-fluorescing band and radio-peak that is principally GA-3. R_f 0·19: a fluorescing band and radio-shoulder that showed no bioactivity and did not correspond to authentic GA-5 or GA-6. Probably a non-gibberellin. R_f 0·38; a blue-fluorescing band and radio-peak that corresponds to GA-4 + GA-7. R_f 0·69: probably GA-9 by fluorescence and comparison with authentic standard.

acid; and (*c*) when authentic ^{14}C-kaurenoic acid was partitioned between 0·5 N sodium carbonate and ethyl acetate, it was found in the organic phase. In longer runs (after 15 days) the kaurenoic acid had been completely metabolized as shown by absence of radioactivity or fluorescence at the R_f value for kaurenoic acid on TLC plates of the neutral fraction B.

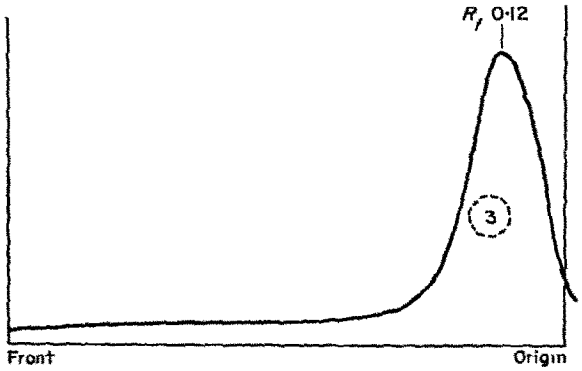


FIG. 3. PREPARATIVE TLC OF THE ACIDIC FRACTION OF *Gibberella fujikuroi* AFTER FEEDING WITH (−)-17-^{14}C-KAURENOIC ACID.

Scan 5: 7-day feed after 3-day growth; purified acidics; charge, 2·77 μc; range, 100,000. Authentic standard: GA-3, R_f 0·13. Analysis: R_f 0·12; almost exclusively GA-3; radio-peak is GA-3 but additional fluorescent spots are visible on the TLC plate.

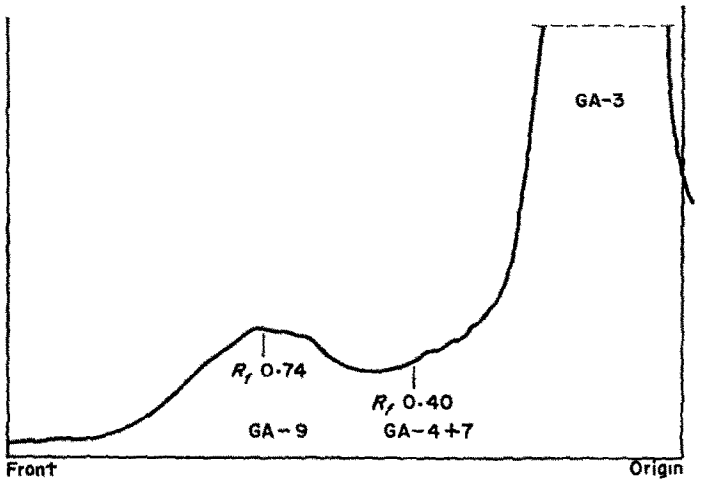


FIG. 4. PREPARATIVE TLC OF THE ACIDIC FRACTION OF *Gibberella fujikuroi* AFTER FEEDING WITH (−)-17-^{14}C-KAURENOIC ACID.

Scan 4: Same as scan 5, but range 10,000 (10 × sensitivity).

It is also to be noted (Table 1) that after longer feeding times the radioactivity in the mother liquors A increases, probably as a result of metabolic degradation of the gibberellins into water-soluble compounds.

The purified acidic fraction E from the 4-hr run was separated on preparative (1 mm thick) TLC plates with solvent system 1. An authentic GA-3 standard was run on the same plate, and a light 5% ethanolic sulfuric acid spray disclosed several blue-fluorescing (u.v.)

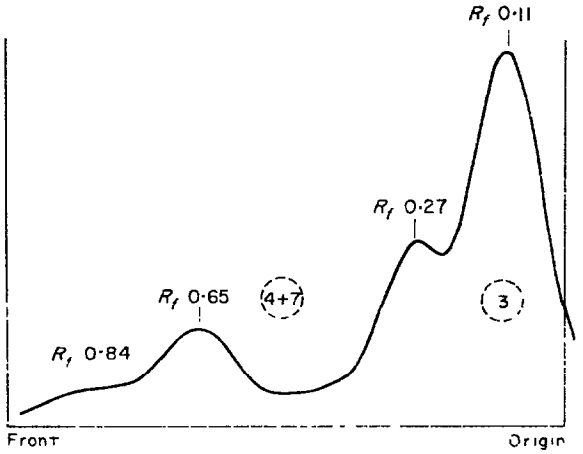


Fig. 5. Preparative TLC of the acidic fraction of *Gibberella fujikuroi* after feeding with (−)-17-^{14}C-kaurenoic acid.

Scan 10: 15-day feed after 3-day growth; purified acidics; charge, 3·74 μc, range, 100,000. Authentic standards: GA-3, R_f 0·11, GA-4+7, R_f 0·51. Analysis: R_f 0·11, GA-3 (isolated and recrystallized to constant specific activity). R_f 0·27; rechromatography showed that this zone did not correspond with GA-5 or GA-6. The radio-peak may be due to a non-gibberellin. R_f 0·65; unknown; no fluorescence or bioactivity. R_f 0·84; probably GA-9 by comparison with authentic standard.

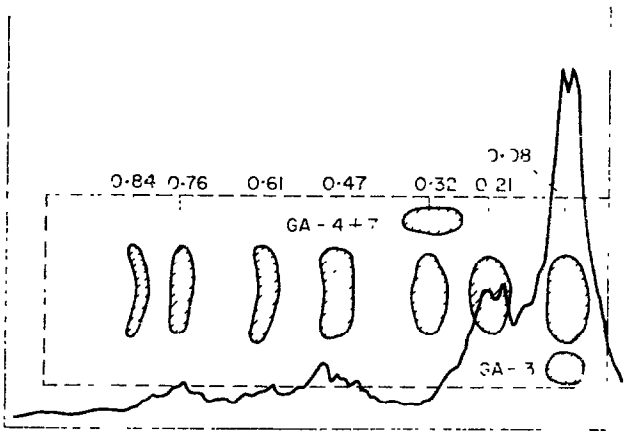


Fig. 6. Preparative TLC of the acidic fraction of *Gibberella fujikuroi* after feeding with (−)-17-^{14}C-kaurenoic acid.

Scan 71: 15-day feed after 3-day growth. Control plate, 0·25 mm thick silica gel-G. Authentic standards: GA-3, R_f 0·08; GA-4+7, R_f 0·32. Spots outlined are visible fluorescent areas in 15 cm run, shown in outline (dotted line). Radio-scan shown as superimposed curve. Analysis: R_f 0·08; GA-3 corresponding to authentic standard. R_f 0·21; unknown radio-peak and fluorescence. R_f 0·32; GA-4+7. No radio-peak, but fluorescent band corresponding to authentic standard. R_f 0·47; unknown radio-peak and fluorescent band. R_f 0·61; red fluorescing non-gibberellin. R_f 0·76; GA-9. Eluted and rechromatographed to show complete correspondence to authentic standard. R_f 0·84; blue fluorescing but not bioactive.

bands. A radio-scan of the plate revealed radioactivity in the GA-1+GA-3 region (these two gibberellins have nearly the same R_f value in this solvent system). However the major radio-peak appeared where a broad blue-fluorescing band, corresponding to GA-4+GA-7

was visible (Fig. 1). A third radio-peak appeared at a higher R_f value corresponding to GA-9. On purification this area showed fluorescence and a weakly positive growth response in a d-5 assay; a study of this material is described in the sequel.

The silica gel zones located by these means were scraped from the plate, extracted with ethanol, and rechromatographed (TLC) in two ways: preparatively (on 1 mm plates) for further purification; or qualitatively (on 0·25 mm plates) for comparison with specimens of authentic gibberellins.

The same general procedure was followed for the 2-, 7-, 15-, and 35-day feeds (Figs. 2–7). In some cases, zones were located, without spraying, by reference to a control plate carrying authentic gibberellin.

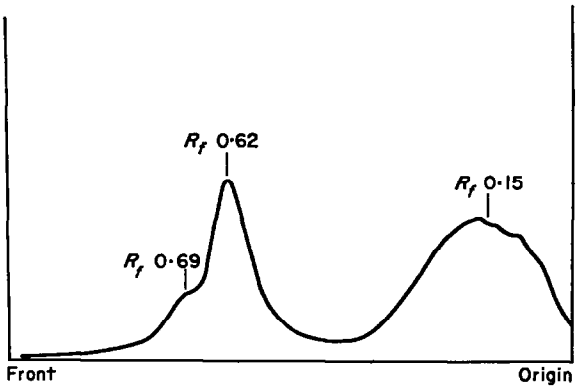


FIG. 7. PREPARATIVE TLC OF THE ACIDIC FRACTION OF *Gibberella fujikuroi* AFTER FEEDING WITH (−)-17-^{14}C-KAURENOIC ACID.

Scan 34: 35-day feed after 3-day growth; purified acidics; charge, 3·83 μc; range, 100,000. Analysis: R_f 0·15; a broad blue-fluorescing zone containing principally GA-3. R_f 0·62; a red-fluorescing zone showing weak bioactivity possibly due to trailing from a bioactive area. Probably not a gibberellin. R_f 0·69; Possibly GA-9; this shoulder or peak is consistently observed in various feeding experiments.

Gibberellins A-4 and A-7

In solvent system 1, GA-4 and GA-7 are not separated but run together at about R_f 0·37 (TLC); this value is subject to some variation according to differences in various sets of TLC plates. This GA-4 + GA-7 zone in the feeding experiments always appears as a broad band or elongated spot, indicating the presence of several components (Fig. 1).

The GA-4 + GA-7 standard used in most of this work was a natural mixture isolated from *Fusarium moniliforme* (Saw.),[13] the identity of which as a mixture of GA-4 and GA-7 has been established by direct comparison with authentic specimens.

In the radio-scan of the TLC plate of the purified acidics E of the 4-hr feed, the highest radioactivity was found to occur at the GA-4 + GA-7 zone (Fig. 1). After a 2-day feed this radio-peak was still present but was considerably diminished, the major peak now appearing in the GA-1 + GA-3 zone (Fig. 2). After the 7-day feed the GA-4 + GA-7 radio-peak has nearly disappeared (Fig. 3), but could be observed as a weak shoulder in a radio-scan at increased sensitivity (Fig. 4). The radioactivity eluted after 15- and 35-day feeds recorded in Table 2 may be due to trailing or to the small amount of GA-4 + GA-7 residual radioactivity.

After 15 and 35 days, the GA-4 + GA-7 radio-peak does not reappear (Figs. 5–7) although the blue-fluorescent band or spot corresponding to authentic GA-4 + GA-7 could always be seen. This absence of radioactivity and the apparent presence of these components can be

interpreted to mean that GA-4 and GA-7 derived from labeled kaurenoic acid has undergone alteration in the course of time, eventually disappearing, to be replaced by material synthesized from unlabelled precursors by normal biosynthetic processes.

In order to establish definitely the presence of radioactive GA-4 and GA-7 in the microbiological reaction sequence, the material from the 4-hr feed, which contained the highest amounts of these gibberellins in radioactive form, was investigated in further detail. The broad GA-4 + GA-7 zone was eluted from the preparative TLC plate and rechromatographed in the same solvent system (No. 1). Two clearly separated blue-fluorescing bands at R_f 0·32 and 0·45 were observed. The slower-moving band (0·32) corresponded to the authentic specimen of GA-4 and GA-7 when compared on a chromatoplate, and had a total radio-

TABLE 2. SUMMARY OF DISTRIBUTION OF ACTIVITY IN GIBBERELLINS A-4, A-7, A-1 AND A-3

Feeding time[a] (days)	GA-4+7 zone (μc)	GA-4+7 (% of E)[b]	R_f[c]	Radioscan	Bioassay[d] Growth	Control	Response
Gibberellin A-4 and A-7							
4 hr	0·52	25	0·32	Strong peak	57·7 ± 2·3	36·7 ± 1·6	-
2	0·21	10	0·38	Medium peak	56·3 ± 2·2	34·6 ± 3·1	-
7	Not eluted		0·48	No peak	Not eluted		
15	0·024	0·6	0·53	No peak	55·1 ± 1·0	39·0 ± 1·6	
35	0·11	2·7	0·44	No peak	46 ± 2·6	34·6 ± 3·1	-
Gibberellin A-1 and A-3							
4 hr	0·25	12	0·11	Medium peak	81·2 ± 4·7	36·7 ± 1·6	
2	1·40	67	0·09	Strong peak	65·7 ± 2·0	34·6 ± 3·1	-
7	0·98	35[e]	0·13	Strong peak	92·5 ± 6·5	39·0 ± 1·5	-
15	1·87	50	0·11	Strong peak	112·5 ± 6·1	39·0 ± 1·5	
35	2·72	71[f]	0·15	Strong peak	103·4 ± 5·3	34·6 ± 3·1	-

[a] All feeds followed 3 days of growth except the 4-hr feed which followed 7 days of growth (Table 1).

[b] Purified acidics E (Table 1) charged on the initial preparative plate.

[c] These values (solvent system 1) are taken from fluorescent spots and/or corresponding radiopeaks compared with authentic gibberellin standards. Variations in plates caused varying R_f.

[d] Bioassay on d-5 of a 0·01 ml/plant aliquot of standard solutions brought to 10 ml with ethanol.

[e] Elution from the silica with acetone gave poor recovery of activity. The 0·98 μc represents 99 per cent of the total radioactivity actually recovered from this TLC plate.

[f] Radioactive GA-1 + 3 was decreasing at this time and the high R_f was due to a broaded fluorescence and radiopeak, as a radioactive catabolic component with an R_f slightly faster than GA-1 + 3 was present.

activity of 0·52 μc, or 25 per cent (Table 2) of the total activity of the aliquot of fraction E added to the original TLC plate. This area thus contained more radioactivity than any other of those eluted from the preparative chromatoplate of the 4-hr run, and represented the major radioactive component. A bioassay was positive.

The separated R_f 0·32 zone could not be further resolved on a 1 mm thick chromatoplate using solvent system 3. However, an aliquot of this zone run on a qualitative (0·25 mm) plate showed a clear separation of two spots: GA-4 at R_f 0·63 and GA-7 at R_f 0·49. These spots corresponded exactly (by fluorescence) with the two spots given by an authentic GA-4 + GA-7 standard, and gave two radio-peaks of about equal intensity when scanned in the strip counter.

It can be concluded that GA-4 and GA-7 are on the biosynthetic pathway from kaurenoic acid to the more highly oxidized gibberellins, since radioactivity in this zone appears early

and decreases as the growing time of the culture increases. Moreover, since GA-7 is more highly oxidized than GA-4 in having a 3,4- double bond, and an oxidative overall metabolic pathway is indicated by the present study, it can be suggested that GA-4 is a metabolic precursor of both GA-7 and GA-3 (Scheme 1).

The faster-moving band at R_f 0·45 (see above) observed in the purification of the crude GA-4 + GA-7 zone contained a high percentage of the total radioactivity of the acidic fraction (0·21 μc, 10 per cent of E) and bioassayed positive, but it did not correspond to any of the known gibberellins 1 to 9 (by direct comparison when specimens were available, by literature R_f values in other cases).

A fraction called "B" by Jones[13] is an unidentified natural bioactive material isolated from *Fusarium moniliforme*.* This was found to be identical with the R_f 0·45 material by direct chromatographic comparison. The nature of this material is still unknown, but its appearance in the 4-hr feed as a radioactive component, and its disappearance in longer feeding studies suggest that it, too, is an intermediate metabolite in the kaurenoic acid-gibberellin pathway.

Gibberellins A-1 and A-3

On thin-layer chromatograms in solvent system 1, GA-1 and GA-3 are not separated and appear at about R_f 0·10–0·15.[14] Separation can be effected with solvent system 2, in which GA-1 has R_f 0·31 and GA-3 has R_f 0·25.

TABLE 3. CRYSTALLIZATION OF GA-1* TO CONSTANT SPECIFIC ACTIVITY

Crystallization	Amount removed for counting (mg)	Activity (μc/mg × 10^3)
1	3·0	1·90
2	3·1	1·14
3	3·5	0·81
4†	2·1	0·78
5‡	3·0	1·11
6	2·9	0·82
7	2·8	0·78

Estimated total activity in GA-1 = 0·025 μc.

* GA-1 zone, 4-hr feed, eluted from TLC, 4·2 mg; plus GA-1, authentic specimen (Abbott), 28·1 mg (Samples of GA-1 lot no. 689-2055 and GA-3, G-1466-152 were obtained from Abbott Laboratories to whom we are grateful).

† Product from recrystallization 4 added to samples removed from 2 and 3 for counting, total recrystallization.

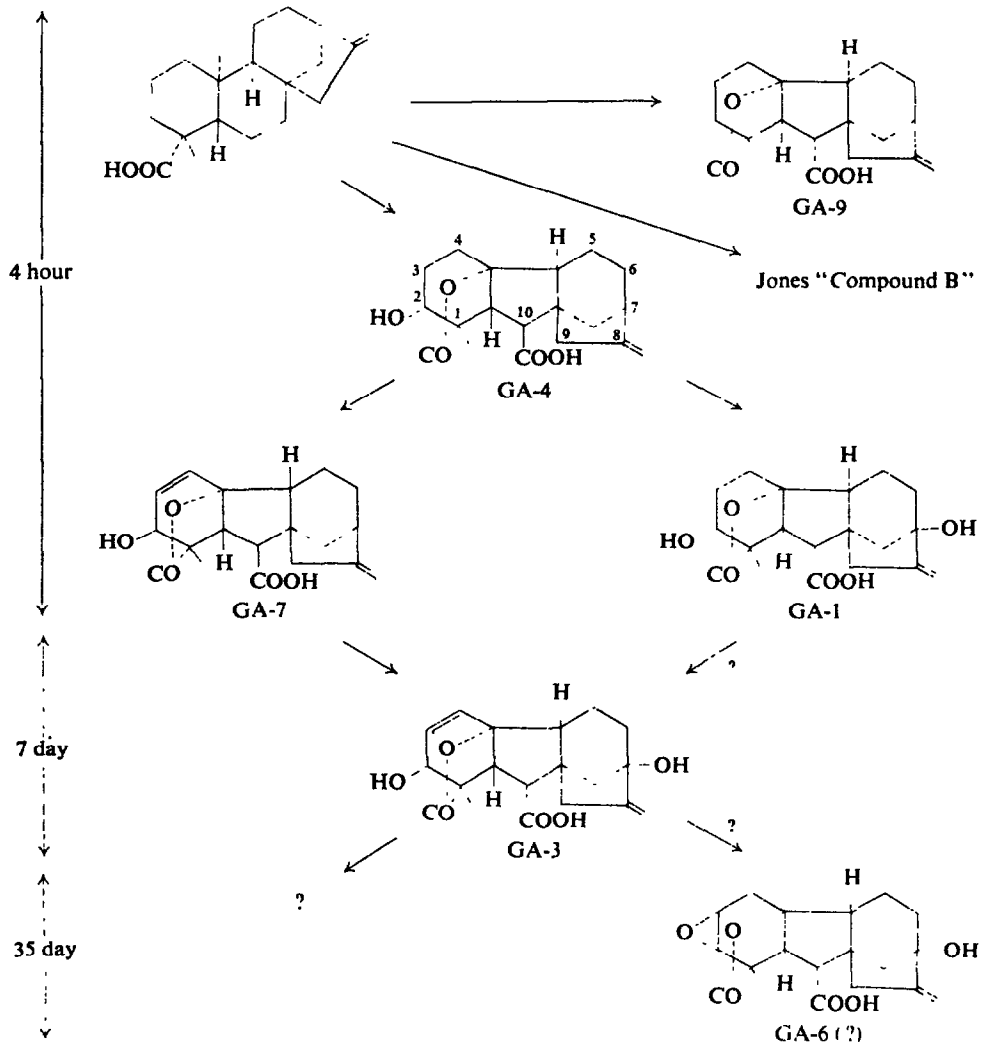
‡ Since this contains material from 2, it has greater activity than 4.

Radio-scans of the TLC plates after a 4-hr feed (Table 2) showed a small but unmistakable radioactive peak at the GA-1 + GA-3 region (Fig. 1). This area was scraped from the plate,

* We are grateful to Mr. Jones for a sample of mixed GA-4+7 and Cpd. B.

[14] J. MACMILLAN and P. J. SUTER, *Nature* **197**, 790 (1963).

extracted, and rechromatographed in solvent system 1 to give a purified mixture of GA-1 + GA-3 (0·25 μc; 12 per cent of E), that bioassayed positive. When an aliquot of this eluate was chromatographed qualitatively on a 0·25 mm plate in solvent system 2, along with authentic GA-3 as a standard, two fluorescing spots appeared, one of which corresponded exactly with GA-3, the other at the correct position for GA-1. The radio-peak of the GA-1



SCHEME 1. SUGGESTED MODE OF BIOSYNTHESIS OF GIBBERELLINS FROM (−)-KAUR-16-EN-19-OIC ACID.

spot was somewhat the stronger of the two. The remaining eluate was then concentrated to dryness and the residue (4·2 mg) diluted with authentic GA-1 (28·1 mg) and recrystallized from ethyl acetate to a constant specific activity of $0{\cdot}78 \pm 0{\cdot}06 \times 10^{-3}$ μc/mg. (Table 3). This represents only 0·5 per cent of the total ^{14}C-kaurenoic acid fed. Thin-layer chromatography of the purified GA-1 in solvent system 2 showed the material to be chromatographically and radiochemically (by scanning) pure, free of GA-3.

After a 2-day feed, a radio-scan of the TLC plate of the acidic fraction (E) showed a major peak in the GA-1 + GA-3 zone, with less intense peaks in the other zones (Fig. 2).

After the 7-day feed a single intense radio-peak at GA-1 + GA-3 dominates the scan (Fig. 3); the other peaks can only be detected by raising the sensitivity of the counter (Fig. 4). Of the total radioactivity recovered from the E fraction in the 7-day run, 99 per cent of it was in the low R_f GA-1 + GA-3 zone. Moreover, when an aliquot of the GA-1 + GA-3 zone eluate was chromatographed along with authentic GA-3 in solvent system 2, two strongly fluorescing spots appeared at R_f values corresponding to GA-1 and GA-3, but only the GA-3 spot was radioactive (by scanning).

Hence, GA-1 appears to have a transient existence as an intermediate metabolite, much like GA-4 and GA-7. Since GA-1 is at a higher oxidation level than GA-4 (GA-1 is hydroxylated at C_7), and since it disappears as a radioactive species while the radioactive GA-3 appears to be increasing at the expense of GA-1, GA-4 and GA-7, it is possible that GA-1 is an intermediate between GA-4 and GA-3 (Scheme 1). This suggests that two metabolic pathways exist for the conversion of GA-4 to GA-3 through GA-1 or GA-7.

TABLE 4. CRYSTALLIZATION OF GA-3 TO CONSTANT SPECIFIC ACTIVITY

Crystallization	Amount removed for counting (mg)	Activity (μc/mg × 10^3)
1	3·0	35
2	4·3	30
3*	3·5	31

Total activity = 1·20 μc or 17% of kaurenoic acid fed. * m.p. 229–230°; m.p. of authentic GA-3 (Abbott specimen), 228–229°; m.p. reported, 233–235°. (GA-3 zone, 15-day feed, eluted from TLC, 5·6 mg, plus GA-3, authentic specimen (Abbott), 29·2 mg.)

The preparative TLC plate prepared from the E fraction of the 15-day feed showed a GA-1 + GA-3 zone that contained both gibberellins, but only the GA-3 component was significantly radioactive, as found by a radio-scan of a qualitative (0·25 mm) plate. The total zone was removed from the plate and extracted to yield 5·6 mg of amorphous residue. To this was added 29·2 mg of authentic, crystalline GA-3 and the whole recrystallized to a constant specific activity of 0·031 μc/mg (Table 2). When this value is used to compute the fraction of the administered radioactivity found in the GA-3, it is found that 1·2 μc or 17 per cent of the kaurenoic acid fed is present in the GA-3. Taking into account the loss in overall recovery (50 per cent, Table 2) from the preparative chromatoplate, it appears that the bioconversion of kaurenoic acid into GA-3 is remarkably efficient. The recrystallized GA-3 had m.p. 229–230° (the authentic specimen had m.p. 228–229°) and was shown by TLC and radio-scanning in solvent system 2 to be radiochemically pure and free of GA-1.

Gibberellin A-9

Gibberellin A-9 has been reported[15] as a product of *Gibberella fujikuroi* (Saw.) Wr. strain ACC 917, but the yield of GA-9 was only 2·7 per cent (0·11 g) of the GA-3 (4·15 g)

[15] B. E. CROSS, R. N. B. GALT and J. R. HANSON, *Tetrahedron* **18**, 451 (1962).

isolated from a culture which had been maintained at pH 6·7–6·8 to maximize GA-9 production. It is clear that GA-9 is made in only very small amounts, but it is probable that it could be detected by chromatographic or bioassay methods in the cultures used in the present studies.

The R_f of GA-9 is reported[14] to be 0·75 on silica gel-G with solvent system 1, and R_f values in the region of 0·7–0·8 have been observed in this study. In many of our TLC plates of the "purified acidics" areas of blue fluorescence and of radioactivity were observed at these R_f values. These were removed and eluted and the concentrated eluates rechromatographed on silica gel-G in solvent system 1. The developed plates were warmed briefly to disclose blue-fluorescing zones which were found to correspond to peaks in radio-scans. These purified zones were again eluted, rechromatographed, and radio- and bio-assayed. The results are summarized in Table 5.

TABLE 5. RECHROMATOGRAPHY OF PRESUMED GA-9 ZONES

Feeding period (days)	R_f Visible	R_f Radio-peak	Radio-assay ($\mu c \times 10^3$)	Bioassay*
4	0·82	0·79	3·7	57·8 ± 2·33†
2	0·81	0·79	2·4	45·8 ± 1·38†
15	0·81	0·81	4·3	45·7 ± 1·44‡
35	0·78	0·76	3·5	42·7 ± 1·53†

* 0·01 ml of 1 ml eluate.
† Control 37·7–1·79 mm.
‡ Control 36·4–1·84 mm.

On comparing 50 μl aliquots of these samples with authentic GA-9 (sample and standard on the same plate), all gave blue-fluorescing spots corresponding to the authentic material. The approximate correspondence of the radioactivity of the GA-9 zone in the 4-hr, 2-day, 15-day, and 35-day samples suggests that GA-9 is indeed formed from kaurenoic acid and that it is not further metabolized. If this is the case, this result is in agreement with the finding[6] that radioactive GA-9 was not converted into GA-3 by *G. fujikuroi*.

Other Gibberellins

Fifteen days after feeding ^{14}C-kaurenoic acid, new peaks began to appear on radio-scans of chromatoplates of acidic fraction E. One of these, at R_f 0·27 in solvent system 1, corresponds to a blue-fluorescing (gibberellin-like) spot which gave a positive but low bioassay (Figs. 5,6). Reported R_f values for the gibberellins show that GA-5 and GA-6 lie in this region with the solvent system used. These gibberellins have not been found to occur in *Gibberella fujikuroi* Lilly strain M-119. An activity of 0·104 μc (1·5 per cent of the kaurenoic acid fed) was eluted from this zone in a 15-day feed. In a preparative plate of a 35-day feed the new zone (GA-5, 6?) and the GA-1 + GA-3 zone blended into a single unresolved radioactive area (Fig. 7), but on qualitative TLC plates (Table 6) there is a resolution both of fluorescence and of radioactivity. Because of the difficulty of resolution in this region on 1 mm preparative plates, the figures given for the GA-1 + GA-3 zone in Table 2 for the 35-day feed actually include the R_f 0·27 zone as well. The lowered bioactivity of the new constituents is to be noted. Further study of these components was carried out as described in the following.

The R_f values 0·31 for GA-5 and 0·25 for GA-6 have been reported for TLC on silica gel-G with solvent system 1.[14] This general area was eluted from preparative TLC plates of the "purified acidics". An aliquot was rechromatographed on 0·25 mm plates along with authentic GA-5 and GA-3 standards and the plates subjected to the 5% ethanolic sulfuric acid reagent and to radioscanning (Table 6). No correlation of fluorescence and radioactivity could be established between GA-5 and the plates of the 2-, 15-, and 35-day feeds, and no slower-moving component (GA-6) was observed in any of the plates. The appearance of radioactivity in the GA-5, GA-6 region in the 2-day plate was not accompanied by visible fluorescence or by bioactivity of the zone. These results indicate that GA-5 and GA-6 are not present in the cultures and that the radioactivity in the zone corresponding to the GA-5, 6 R_f value is due to some other material whose identity is still unknown.

Other radio-peaks and fluorescent zones appeared at higher R_f values in 35-day experiments, but these constituents showed low activity in the bioassay, and no further study has yet been devoted to them.

TABLE 6. SUMMARY OF TLC EXAMINATION OF GA-5, GA-6 ZONES

Feeding period (days)	Compound	R_f	Fluorescence	Radio-scan
2	GA-3 standard	0·15	Blue	
	GA-5 standard	0·55	Blue	
	GA-5+6 zones*	0·52	None	Strong peak
15	GA-3 standard	0·13	Blue	
	GA-5 standard	0·53	Blue	
	GA-5+6 zones	0·63	Weak blue	Peak
35	GA-3 standard	0·15	Blue	
	GA-5 standard	0·49	Blue	
	GA-5+6 zones†	0·15	Blue	Strong peak
		0·64	Weak blue	Weak peak

* Lack of fluorescence and negative bioassay indicate that this component is not GA-5 or GA-6.

† The zone eluted from the preparative plate was wide enough to include GA-1, 3, 5 and 6, but evidently the main component was GA-3.

EXPERIMENTAL

Radioactivity measurements were carried out with the use of a Nuclear-Chicago Scintillation Spectrometer, Model 720. The solvent used was dioxan containing 0·7% of 2,5-diphenyloxazole (PPO), 0·05% 2,2′-p-phenylene-bis-(5-phenyloxazole) (POPOP), and 5% naphthalene. Radioscanning of the chromatographic plates was carried out on a Vanguard Automatic Chromatogram Scanner, Model 880, with a glass-plate attachment.

Preparative plates carried a 1 mm thick layer of Merck silica gel-G and qualitative plates used for comparison and identification had the usual 0·25 mm thick layers of Merck silica gel-G. Plates were dried for 2 hr at 100°.

The solvent systems used for TLC were the following: Solvent system 1: isopropyl ether–acetic acid (95:5). Solvent system 2: isopropyl ether–acetone–acetic acid (90:30:1). Solvent system 3:[16] carbon tetrachloride–acetic acid–water (8:3:5), were shaken together

[16] T. KAGAWA, T. FUKINBARA and Y. SUMIKI, *Agr. Biol. Chem.* **27**, 598 (1963).

and separated. The TLC plates were allowed to equilibrate with the aqueous phase overnight, and then developed with a mixture of the lower phase (8 parts) and ethyl acetate (1·6 parts). Solvent system 4: benzene–ether (1:1).

Synthesis of (−)-17-^{14}*C-kaur*-16-*en*-19-*oic Acid*

Methyl iodide −^{14}C (111 mg, nominal 5 mc)* was diluted with 205 mg of unlabeled methyl iodide in benzene, and the solution added to a benzene solution of 670 mg of triphenylphosphine. After 5 days the ^{14}C-triphenylmethylphosphonium iodide (960 mg, 2·38 m-mole) was collected and dried, then suspended in 10 ml of dry, freshly distilled tetrahydrofuran (THF). Butyllithium (3·5 ml of a 1·67 N solution in hexane; 5·1 m-moles) in 15 ml of THF was added dropwise with stirring until the solution was clear and had assumed the characteristic orange color.† After 5 min the solution of the Wittig reagent was treated with a solution of 225 mg (0·74 m-mole) of (−)-17-norkauran-16-on-19-oic acid in 10 ml of THF and the mixture stirred overnight and then heated under reflux for 5 hr. The THF was evaporated, water was added to the residue, the aqueous solution was carefully acidified and extracted with ether, and the ether dried and evaporated. The oily residue (580 mg) was chromatographed on silica, from which elution with benzene–ether (95:5) afforded (−)-17-^{14}C-kaur-16-en-19-oic acid (100 mg). This was recrystallized from methanol to give 70 mg of the pure acid, m.p. 169–170°, identical (mixed m.p.) with an authentic specimen of unlabeled material.

The radioactivity of the ^{14}C-kaurenoic acid was found to be 6·04 μc/mg, or 1·82 mc/m-mole; a radio-scan of a TLC plate of the compound showed only the single radioactive spot.

Growth and Extraction of Gibberella fujikuroi

De Long culture flasks (125 ml) with Morton stainless-steel closures were charged with 50 ml of the 10% ICI medium, sterilized and inoculated with *Gibberella fujikuroi* Lilly strain M-119. After 3 days on a rotary shaker at 26° the cultures became pink and on this day or later, as shown in the figures, feeding was initiated. As an example, in the 4-hr feed, 10 μc (1·7 mg) of ^{14}C-kaurenoic acid in 1·0 ml of ethanol was distributed equally between two flasks. After the time specified in Table 1, the two flasks were processed simultaneously as samples 1 and 2.

The culture medium containing the pink mycelium was filtered by suction and washed with water. The filtrate was brought to pH 2·5 with 2 N HCl and extracted with 3 × 25 ml of ethyl acetate, the aqueous mother liquors (A) being radio-assayed. Acidics were extracted from the ethyl acetate with 4 × 10 ml of 0·5 M Na_2CO_3, the residual ethyl acetate containing the neutrals (B). The Na_2CO_3 solution was brought to pH 2·5 with 2 N HCl, extracted with 3 × 25 ml of ethyl acetate, and the aqueous miscellaneous (C) layer radio-assayed. The ethyl acetate solution of crude acidics (D) was concentrated to 1 ml, passed through a column containing 0·5 g of Merck silica gel-G, and the column was washed well with 60–75 ml of ethyl acetate and finally with 20 ml of absolute ethanol. In every case a dark radioactive residue remained on the column and was discarded. The ethyl alcohol-ethyl acetate effluent contained the "purified acidics" E. Recovery of activity in these acidics was 38–56 per cent (Table 1)

* The initial radioactivity was not checked. Radioactivity measurements were made on the final crystalline product.

† As reported by previous workers and as ascertained in trial runs, when only the theoretical amount of butyllithium was used the yield of final product was low.

Thin-layer Chromatography

The solution of purified acidics E was separated by preparative TLC using a control plate which was developed in the tank at the same time. Solvent 1 was used for the first separation and all plates were developed for 15 cm. The control plate was sprayed with 5% H_2SO_4 in ethanol, heated at 100° for 10 min to detect the u.v. fluorescent gibberellins. These spots were used to locate zones on the preparative plate. Later it was found that lightly spraying the preparative plate did not destroy the gibberellins to any appreciable extent, and provided a more accurate means for locating the fluorescing bands. At least one authentic gibberellin was run on each plate since R_f values vary considerably on plates prepared at various times. Zones were also located by a radio-scan of the TLC plate. The radiopeaks almost always corresponded to fluorescent bands, but the reverse was not so, since there were many fluorescent spots, especially after 35 days, which showed little or no radioactivity. The selected zones (usually 0·7–2 cm on each side of the R_f value) of the silica were removed from the plate, eluted with acetone or absolute ethanol, radioassayed, bioassayed and further identified. Purification was effected by rerunning the eluted gibberellins on a second TLC with the same solvent system or one that would separate components that have similar R_f values. GA-1 could be separated from GA-3 using solvent system 2 which gave a 1-cm separation in a 15-cm run. GA-4 and GA-7 are difficult to separate preparatively but can be qualitatively identified using solvent system 3 which gives a 2-cm separation in a 15-cm run.

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