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STUDIES ON THE BIOSYNTHESIS OF GIBBERELLINS—II.

THE BIOSYNTHESIS OF GIBBERELLINS FROM (−)KAURENOL, AND THE CONVERSION OF GIBBERELLINS ^{14}C-GA-4 AND ^{14}C-GA-7 INTO ^{14}C-GA-3 BY *GIBBERELLA FUJIKUROI*

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Abstract—The formation of radioactive gibberellins from (−)kaurenol ((−)kaur-16-en-19-ol) in cultures of *Gibberella fujikuroi* has been found to resemble that from (−)kaurenoic acid. Evidence for an intermediate step in the pathway to gibberellic acid (GA-3) has been obtained in the observation that a radioactive GA-4+ GA-7 fraction obtained from a kaurenol-fed culture gave rise to radioactive G-3 when fed to a second culture.

INTRODUCTION

IN AN earlier paper[1] evidence was presented to show that (−)kaur-16-en-19-oic acid serves as a precursor to gibberellins in cultures of *Gibberella fujikuroi*. The considerable degree of probability that kaurenoic acid lies on a pathway proceeding from (−)kaurene and leading eventually to the gibberellins is supported not only by our observations[1] but also by the demonstration that (−)kaurene itself is a gibberellin precursor[2] and has gibberellin-like activity in the dwarf maize mutant *d*-5;[3] by the isolation of numerous intermediate products of an oxidative sequence leading from kaurene toward the gibberellins;[4] the gibberellin-like activity of kaurenol and kaurenoic acid in the *d*-5 mutant;[5] and by the demonstration that kaurenol is present along with gibberellins in endosperm nucellus of *Echinocystis macrocarpa* Greens.[6] That (−)kaurenol, like (−)kaurenoic acid, gives rise to gibberellins in *G. fujikuroi* cultures, has been reported[6,7] and has been confirmed in a part of the study described in this paper.

In the earlier experiments,[1] evidence was adduced to indicate that the various gibberellins produced by the fungus are formed in a sequence in which successive oxidative changes led through GA-4 and/or GA-7 to GA-1 and finally to GA-3. While this course is consistent with an overall oxidative transformation of kaurene to gibberellins in a sequence in which the earlier stages clearly involve oxidations at C-19, C-6, C-7, and C-20,[4] the observations of the time-course of changes described in our first paper were, while compelling, not conclusive evidence for the sequence of transformations among the gibberellins themselves. We have

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[1] T. A. GEISSMAN, A. J. VERBISCAR, B. O. PHINNEY and G. CRAGG, *Phytochem.* **5**, 933 (1966).
[2] B. E. CROSS, R. H. B. GALT and J. R. HANSON, *J. Chem. Soc.* 295 (1964).
[3] B. O. PHINNEY, P. R. JEFFERIES, M. KATSUMI and C. A. HENRICK, *Plant Physiol.* **39**, Suppl. XXVII (1964).
[4] B. E. CROSS, R. H. B. GALT, J. R. HANSON, P. J. CURTIS, J. F. GROVE and A. MORRISON, *J. Chem. Soc.* 2937 (1963).
[5] M. KATSUMI, B. O. PHINNEY, P. R. JEFFERIES and C. A. HENRICK, *Science* **144**, 859 (1964).
[6] J. GRAEBE, D. T. DENNIS, C. D. UPPER and C. A. WEST, *J. Biol. Chem.* **240**, 1847 (1965).
[7] R. H. B. GALT, *J. Chem. Soc.* 3143 (1965).

now been able to provide further support for the earlier suggestions that GA-4 and GA-7, which are at lower oxidation levels than GA-3, are indeed transformed into the latter in cultures of *G. fujikuroi.*

RESULTS AND DISCUSSION

The acidic fractions, containing the gibberellins but lacking kaurenol, isolated from the two culture media of *Gibberella fujikuroi* after subsequent growth for 2 and 14 days, respectively, were separated on TLC plates (Table 1). Figures 1 and 2 show the location of radio-

TABLE 1. SUMMARY OF FEEDING EXPERIMENTS*

Feeding time:	2 days	14 days	7 days
Days growth before feeding:	3 days	3 days	7 days
Sample fed:	17-^{14}C-kaurenol	17-^{14}C-kaurenol	^{14}C-gibberellins A-4 and A-7
Activity of sample fed*	19·0	19·0	0·076
Activity in mother liquors (A)†	1·33	1·40	0·005
neutral material (B)	3·73	2·76	0·000
aqueous miscellaneous (C)	0·40	0·49	0·021
crude acidics (D)	8·22	7·90	0·042
total recovery	13·68	12·55	0·068
per cent recovery	72	66	90
purified acidics (E)	6·03	7·30	0·038
(E) as per cent of feed	32	38	51

* Figures are μc of radioactivity.
† Fractions A, B, etc. correspond to workup described earlier.[1]

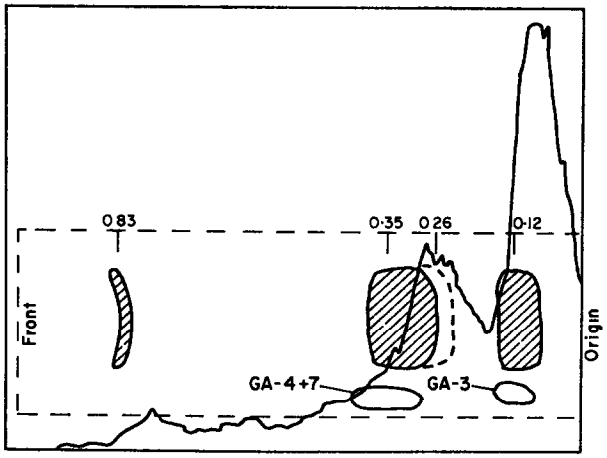

FIG. 1.

SCAN NO. 81: 2-DAY FEED OF 17-^{14}C-KAURENOL AFTER 3-DAY GROWTH; PURIFIED ACIDICS (E); CONTROL PLATE; SOLVENT SYSTEM NO. 1.

Authentic standards: GA-3 R_f 0·12, GA-4+7 R_f 0·35.

Analysis: R_f 0·08–0·12, blue fluorescent and radioactive GA-1 and GA-3; R_f 0·26, a radiopeak roughly corresponding to an unidentified red band superimposed on a blue fluorescence; R_f 0·35, authentic GA-4+7 standard. The blue fluorescing band at R_f 0·32 overlaps with the red band preceding it; R_f 0·83, blue fluorescing GA-9.

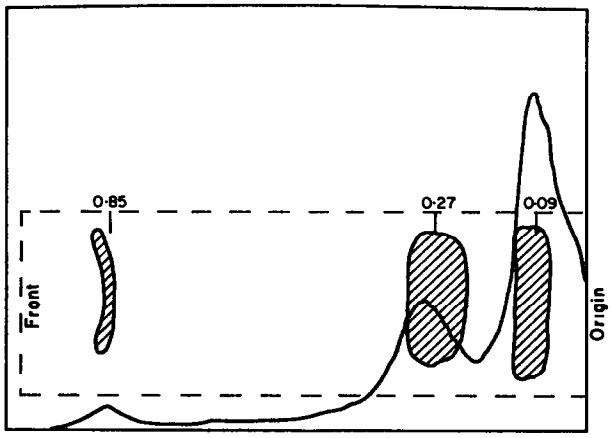

FIG. 2.

SCAN NO. 82: 2-DAY FEED OF 17-^{14}C-KAURENOL AFTER 3-DAY GROWTH; PURIFIED ACIDICS (E); CHARGE 6·0 μC; RANGE 100,000; SOLVENT SYSTEM NO. 1.

Authentic standards: Run with a control plate (Fig. 1) containing GA-3 and GA-4+7 standards.

Analysis: R_f 0·09, a blue fluorescing band and radiopeak comprising a mixture of GA-1 and GA-3; R_f 0·27, a red fluorescing band corresponding to a radiopeak at R_f 0·29 of unknown content. This peak also contains some radioactive GA-4+7 in the leading edge; R_f 0·85, a weak blue fluorescing band with a corresponding radiopeak in the GA-9 area.

activity and fluorescent regions of a control plate (Fig. 1) and a preparative plate of the same material (Fig. 2) for the 2-day experiment. It is apparent that a strong radio peak and a fluorescent zone at low R_f correspond to the GA-3 standard. The strong radio peak at R_f 0·29 (preparative) and 0·26 (control) contains some radioactive GA-4+GA-7 but is largely due

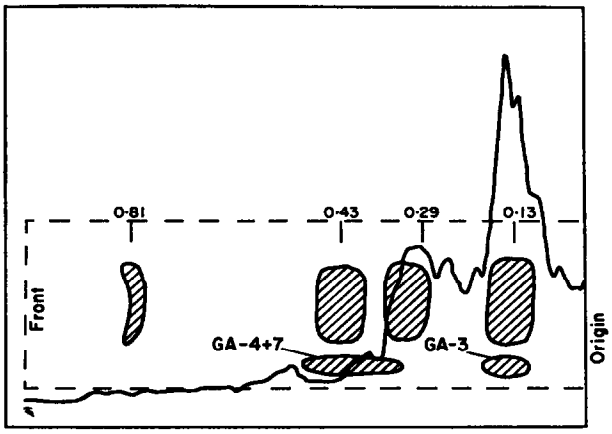

FIG. 3.

SCAN NO. 87: 14-DAY FEED OF 17-^{14}C-KAURENOL AFTER 3-DAY GROWTH; PURIFIED ACIDICS (E); CONTROL PLATE; SOLVENT SYSTEM NO. 1.

Authentic standards: GA-3 R_f 0·14, GA-4+7 R_f 0·43.

Analysis: R_f 0·13, a radiopeak corresponding to a blue fluorescence containing radioactive GA-1 and GA-3; R_f 0·29, a radiopeak corresponding to none of the gibberellins; R_f 0·43, GA-4+7 zone fluorescing blue; R_f 0·81, blue fluorescing GA-9 area.

to that of another, unidentified, material. The low R_f (0·09) zone was eluted and rechromatographed with authentic GA-1 and GA-3[8] and shown to contain both of these gibberellins.

The radioscan of the control TLC plate of the 14-day feed is shown in Fig. 3. Again, the strong radio peak of GA-1/GA-3 is present, and the unknown radioactive material at R_f 0·29 is again seen. Very little radioactivity is found at the R_f of GA-4+GA-7, although the presence of these gibberellins as radio-inactive constituents is shown by the fluorescent band that corresponds with the authentic control.

Gibberellins A-2 and A-3

The zones corresponding to the authentic GA-3 standard in the preparative TLC plates of the 2- and 14-day feeds were scraped from the plates and eluted. Positive bioassays on *d*-5 seedlings showed that these zones contained gibberellins. Although GA-1 and GA-3 do not separate satisfactorily under the usual conditions of TLC with any of the solvent systems used, a good separation was achieved with the use of long (60 cm) plates using solvent system 2. Qualitative identification of GA-1 and GA-3 by simultaneous chromatography with the authentic compounds showed that these gibberellins were present in both cultures.

Elution of the separated GA-1 and GA-3 zones from long-plate TLC's, and measurement of the radioactivity of each zone by scintillation counting gave the results shown in Table 2.

TABLE 2. GIBBERELLIN A-1 AND A-3 SEPARATION ON LONG TLC PLATES OF GA-1 AND GA-3 ELUTED FROM PREPARATIVE PLATE

	2-day kaurenol feed	14-day kaurenol feed	7 day GA-4+GA-7 feed
Charge on plate††	$1{\cdot}14\times10^{-2}$	$1{\cdot}98\times10^{-2}$	$6{\cdot}7\times10^{-4}$
Distance to front, cm*	46·5	51·0	50·7
R_f GA-1†	0·46	0·43	0·34
R_f GA-3	0·40	0·37	0·28
Activity in GA-1 zone‡ ††	$0{\cdot}45\times10^{-2}$	$0{\cdot}55\times10^{-2}$	$0{\cdot}84\times10^{-4}$
Activity in GA-3 zone††	$0{\cdot}32\times10^{-2}$	$0{\cdot}97\times10^{-2}$	$2{\cdot}91\times10^{-4}$
Per cent of charge counted§	67	77	56**
Ratio of activity GA-3/GA-1	0·7	1·8	3·5

* Run overnight, solvent system 2.

† Variations in preparation of plates and in conditions of development account for variation in absolute values of R_f.

‡ Fluorescing zone scraped off directly into scintillation solution and counted.

§ As the charge placed on the plate was counted under conditions different from those used for counting the zones, the recovery of radioactivity is of dubious significance.

** Radioactivity was present in the zones preceding and following the GA-1 and GA-3 zones. A total of about 13 per cent of the activity charged onto the plate was found in these other regions, making the total recovery of activity about 70 per cent.

†† All activity figures are in microcuries.

The higher GA-3/GA-1 ratio in the 14-day feed compared with the 2-day feed supports the suggestion made earlier[1] that the synthetic sequence leading from kaurene derivatives to the gibberellins involves progressive oxidation steps.

[8] The authors are grateful for the gift of samples of GA-1 (lot No. 689-2055) and GA-3 (lot No. G-1466-152) from Abbott Laboratories.

Gibberellins A-4 and A-7

The GA-4+GA-7 zones (Figs. 1, 2 and 3) removed from the preparative TLC plates of the 2- and 14-day feeds were eluted, further purified by TLC, and identified by TLC comparisons with authentic material. Bioassays for the eluted materials were positive.

The original eluate from the GA-4+GA-7 zone of the 2-day feed contained a considerable amount of radioactive impurity from the peak at R_f 0·29 (Figs. 1 and 2). This impurity could be separated from the GA-4 + GA-7 by preparative rechromatography with solvent system 1; the recovered GA-4+GA-7 showed (TLC, solvent system 3) only two fluorescing bands, corresponding to authentic GA-4 and GA-7 and coinciding with a poorly resolved radio-peak (Fig. 4). Removal of the radioactive material by separating the fluorescent zones and counting the two portions gave 1950 dpm for the GA-4 region and 1980 dpm for the GA-7 region, or a

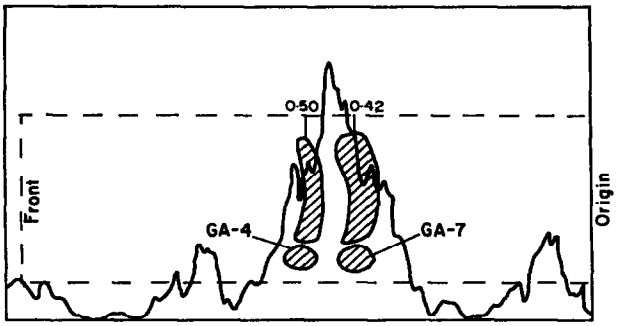

FIG. 4.

SCAN NO. 91: 2-DAY FEED OF 17-^{14}C-KAURENOL AFTER 3-DAY GROWTH; REPURIFIED GA-4+7 ZONE; CONTROL PLATE; SOLVENT SYSTEM NO. 3.

Authentic standards: GA-7 R_f 0·42, GA-4 R_f 0·51.

Analysis: R_f 0·42, blue fluorescent band and radioactivity corresponding to authentic GA-7; R_f 0·50, blue fluorescent band and radioactivity corresponding to GA-4.

This TLC and radioscan is a purity check on the material fed to *G. fujikuroi*. Although other minor radiopeaks are visible there were no fluorescent areas other than these two bands.

GA-7/GA-4 ratio of 1·0.[9] The purified GA-4+GA-7 mixture from the second preparative plate (only an aliquot of which was used for identification; see footnote 8) contained a total of 0·118 μc. An 0·076-μc portion of this was used for the feeding experiment described below.

Treatment of the GA-4+GA-7 region of a preparative plate prepared from the 14-day feed in a manner similar to that just described for the 2-day feed led to eventual separation (TLC, solvent system 3) of the GA-4 and GA-7 zones. These showed a radioactivity ratio of GA-7/GA-4 of 1·6. Although the total radioactivity of the GA-4+GA-7 in the 14-day feed was much less than that in the 2-day feed, the increase in the GA-7/GA-4 ratio suggests that GA-4 lies before GA-7 on the synthetic pathway. It is to be emphasized, however, that no absolute significance can be attached to the observed ratios because of the imperfect purifications and separations obtained; the increase in the GA-7/GA-4 ratio from 1·0 to 1·6 does appear, nevertheless, to be indicative of the suggestion made above.

[9] This analytical TLC plate was a thin (0·25 mm) layer, and thus contained very little total activity.

Gibberellin A-9

The blue fluorescent band at R_f 0·83 and 0·81 on the TLC plates of the 2- and 14-day feeds (Figs. 1, 2 and 3) was found to correspond to GA-9 after elution and rechromatography with an authentic specimen. Bioassay of the purified GA-9 was positive.

Feeding of ^{14}C-GA-4+GA-7

A purified fraction of the mixed GA-4+GA-7 from a 2-day feed of ^{14}C-kaurenol (see section headed "Gibberellins A-4 and A-7"), containing 0·076 μc of activity, was fed to a 7-day culture of *G. fujikuroi* and worked up after 7 days of growth. The recovery of radioactivity in the "purified acidics" (E) was 51 per cent of the amount fed (Table 1). This fraction was separated into its components on a preparative TLC plate, the radioscan of which is shown in Fig. 5. Two major radiopeaks were present: one at an R_f of about 0·1, corresponding to GA-1 and GA-3; the other at an R_f of about 0·3, corresponding to GA-4 and GA-7, and

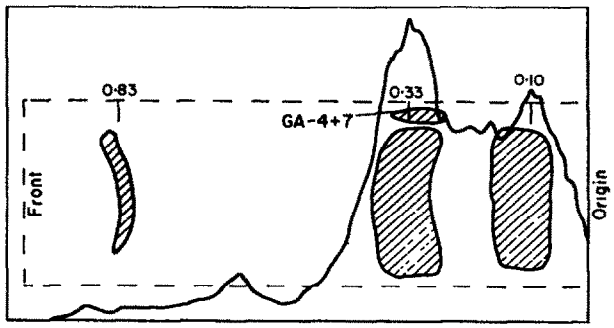

FIG. 5.

SCAN NO. 95: 7-DAY FEED OF ^{14}-C-GIBBERELLIN A4+A7 AFTER 7-DAY GROWTH; PURIFIED ACIDICS (E); CHARGE 0·0383 μC; RANGE 1000; SOLVENT SYSTEM NO. 1.

Authentic standard: GA-4+7 R_f 0·30.

Analysis: R_f 0·10, radiopeak corresponding to blue fluorescence at R_f 0·11 due to GA-1+GA-3; R_f 0·33, radiopeak due to unmetabolized GA-4+7 corresponding to blue fluorescence and authentic standard; R_f 0·83, blue fluorescent band due to GA-9.

presumably representing the administered GA-4 and GA-7 that was not utilized. Of the 0·038 μc of the purified acidic fraction (E) charged on the TLC plate, 0·013 μc was found in the R_f 0·1 zone and 0·014 μc in the R_f 0·3 zone, for a total recovery of 72 per cent of the acidic fraction (E). Re-chromatography of the eluted R_f −0·3-zone along with authentic material showed that GA-4 and GA-7 were present.

The GA-1+GA-3 zone was eluted, and a $6{\cdot}7 \times 10^{-4}$ μc aliquot chromatographed on a 60 cm TLC plate using solvent system 2. The chromatogram showed two well-resolved zones (by fluorescence) corresponding to GA-1 (R_f 0·34) and GA-3 (R_f 0·28). Each of these areas was removed from the plate and counted directly in the scintillation counter (Table 2). The GA-3/GA-1 ratio was 3·5.

This result shows clearly that GA-1/3 are formed from GA-4/7, since the only radioactive substrate in this fungus culture was the GA-4/7 which (Fig. 4) is essentially free of GA-1/3.

Experiments now in progress are directed to the study of the fate of labeled kaurenol and kaurenoic acid in the *d*-5 dwarf mutant of *Zea mays*, for it is not a necessary conclusion, although a reasonable and probable one, that the effect of kaurene derivatives in promoting the growth of the *d*-5 mutant is the result of their conversion into gibberellins in the plant.

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 per cent of 2·5-diphenyloxazole (PPO), 0·05 per cent of 2,2′-*p*-phenylenebis-(5-phenyloxazole) (POPOP) and 5 per cent of naphthalene. Radio-scanning of the chromatographic plates was carried out with a Vanguard Automatic Chromatogram Scanner, Model 880, equipped with a glass-plate attachment.

TLC refers to thin-layer chromatography. Preparative plates carried a 1 mm thick layer of Merck silica gel G, and qualitative plates had the usual 0·25 mm layer of the same adsorbent. The general procedures used for the development, examination and elution of TLC plates are described in an earlier paper.[1] Special long plates, 5 × 60 cm, were used to separate GA-1 and GA-3. Development was carried out overnight, the solvent front ascending 40–50 cm in this time. These plates were given a predevelopment with acetone–acetic acid (100:1) until the gibberellins were concentrated in a thin line about 0·5 cm from the origin. Subsequent development was then carried out with solvent system 2.

Solvent systems: (1) isopropyl ether–acetic acid, 95:5; (2) isopropyl ether–acetic acid–acetone, 90:1:30; (3) carbon tetrachloride–acetic acid–water, 8:3:5, of which the lower phase (8 parts) and ethyl acetate (1·6 parts) were mixed and used for plates pre-equilibrated with the upper phase.

16-Oxo-17-Nor(−)Kauran-19-ol

To a solution of 2 g of (−)kauran-16α,17,19-triol[10] in 150 ml of ethanol was added a solution of 2 g of periodic acid in 5 ml of water. The solution was stirred at room temperature for 3·5 hr, poured into water, and the mixture extracted with ether. The ether extract was washed with aqueous $NaHCO_3$, dried ($MgSO_4$) and evaporated. The product (1·66 g) was recrystallized from aqueous acetone to give the pure ketone, m.p. 155–156° (reported,[11] m.p. 154–157°).

17-^{14}C-Kaur-16-en-19-ol

Methyl iodide-^{14}C (111 mg)[12] was diluted with 222 mg of unlabeled methyl iodide and added to a solution of 600 mg of triphenylphosphine in 25 ml of benzene. After 3 days the triphenylmethylphosphonium iodide (824 mg) was collected, dried, and suspended in 10 ml of dry tetrahydrofuran (THF) under nitrogen. A solution of butyllithium (2·5 ml of a 1·64 N solution in hexane; a 2-molar excess) in 20 ml of THF was added dropwise, with stirring until the solution was clear and had assumed the characteristic orange color. After 5 min, to the solution of the Wittig reagent was added a solution of 185 mg of 16-oxo-17-norkauran-19-ol acetate (prepared from the alcohol with pyridine–acetic anhydride; m.p. 121–123°; found: C, 75·78%; H, 9·75%. $C_{21}H_{32}O_3$ required: C, 75·86%; H, 9·70%) in 10 ml of THF, and the mixture stirred overnight and finally heated under reflux for 3 hr. The solution was evaporated to dryness under reduced pressure and the residue treated with water and ether. The ether solution was washed with aqueous $NaHCO_3$, dried and evaporated. The residue (545 mg) was chromatographed over 40 g of neutral alumina (act. IV), from which elution with benzene gave 70 mg of (−)17-^{14}C-kaur-16-en-19-ol. Recrystallization from aqueous methanol gave 50 mg of the pure kaurenol, m.p. 141–142° (reported,[11] m.p. 141–142°), identical by mixed m.p. with authentic material. The radioactivity, measured on two samples, was 10·88 and 10·94 μc/mg (3·14 mc/mM).

17-^{14}C-Kaurenol Feeding Experiments

Two cultures of *Gibberella fujikuroi* (Lilly strain M-119) were grown for 3 days in 50 ml of 10% ICI medium; and each was then fed with 19 μc (1·75 mg) of (−)17-^{14}C-kaur-16-en-19-ol in 0·5 ml of ethanol. The cultures were harvested 2 and 14 days after feeding and worked up in the manner described earlier[1] to yield fractions A, the ethyl acetate-extracted culture solution; B, neutral materials not removed from the ethyl acetate solution by Na_2CO_3; C, the aqueous solution remaining after ethyl acetate extraction from the acidified Na_2CO_3 solution; D, the acidic material extracted by ethyl acetate from the acidified Na_2CO_3 extract; and E, the "purified" acidic materials obtained by passage of D through a column of silica gel in acetone. Recovered kaurenol was found in B, and gibberellins were found in E, and were separated on preparative TLC plates using solvent system 1. Control plates, containing authentic GA-3 and GA-4 + GA-7 as standards, were run along with the preparative plates. Zones on the preparative plates corresponding to the separate gibberellins were scraped off and eluted with acetone, bioassayed, and further chromatographed for establishment of their identities.

[10] We are grateful to Professor P. R. Jefferies of the University of Western Australia for the specimen of the triol used in this work.

[11] C. A. Henrick and P. R. Jefferies, *Australian J. Chem.* **17**, 915 (1964).

[12] The activity of the methyl iodide was not checked; radioactivity measurements were carried out on the final purified kaurenol.

Gibberellin A-4+7 Feed

A specimen of GA-4+GA-7, isolated from a kaurenol-fed culture of *G. fujikuroi*, and containing 0·076 μc of activity was added to a 50 ml culture of the fungus grown for 7 days in a 10% ICI medium in a Delong culture flask. After 7 days the culture was filtered from the mycelium and worked up in the usual way (Table 1). A control culture grown in the same way yielded a comparable dry weight of mycelium, showing that the fungus growth had proceeded normally. Separation and identification by TLC was carried out as described above, with results described in the section on results.

Bioassays

Each fraction to be assayed for biological activity was dissolved in ethanol and the volume adjusted to 1·4 ml. A 0·2 ml portion of this was added to the first unfolding leaf of seven *dwarf*-5 seedlings. Ten days later the lengths of the first and second leaf sheath were measured and summed for each seedling. The mean and standard error were determined for each set of seven plants. Assay responses were not regarded as positive unless they were 10 per cent or greater than over the controls; i.e. non-treated *d*-5 seedlings.

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