Phytochemistry, 1973, Vol. 12, pp. 2655 to 2659. Pergamon Press Printed in England.

CONVERSION OF GIBBERELLIN A_1 INTO GIBBERELLIN A_3 BY THE MUTANT R-9 OF *GIBBERELLA FUJIKUROI**

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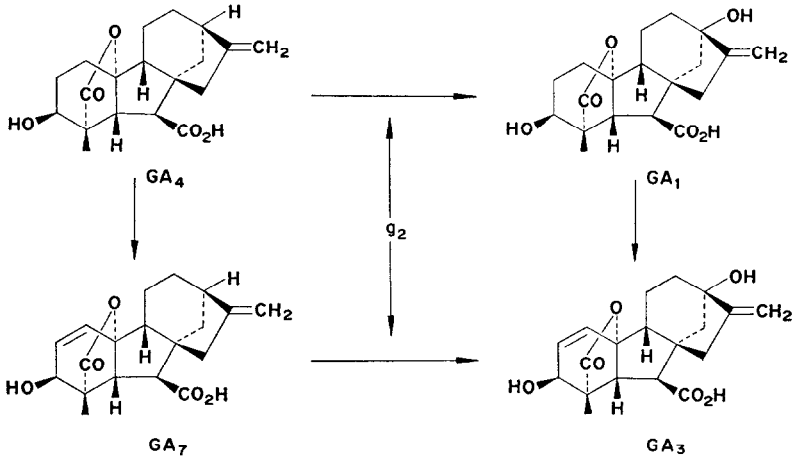
(*Received* 5 *April* 1973. *Accepted* 17 *May* 1973)

Key Word Index—*Gibberella fujikuroi*; fungus; gibberellin biosynthesis; genetic mutants; conversion of GA_1 to GA_3.

Abstract—A mutant R-9 of *Gibberella fujikuroi* has been isolated and shown to be blocked for GA_1 and GA_3 biosynthesis, but not for GA_4, GA_7 and other gibberellins. Cultures of this mutant convert low concentrations of [1,2-$^3H_2$]-GA_1 into GA_3 in a radiochemical yield of 2·7%.

SPECTOR and Phinney[1] reported the first evidence for the genetic control of gibberellin (GA) production in the fungus, *Gibberella fujikuroi*. From genetic and biochemical studies they demonstrated the presence of two non-allelic genes that blocked different steps in the GA biosynthetic pathway. The first gene (g_1) blocked an early step and controlled all GA



SCHEME 1. PROPOSED FINAL STEPS OF GA_3-BIOSYNTHESIS IN *Gibberella fujikuroi*.

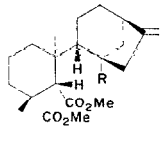
* Part VII in the series "Fungal Products". For Part VI see BEARDER, J. R., MACMILLAN, J. and PHINNEY, B. O. (1973) *Phytochemistry* **12**, 2173.

[1] SPECTOR C. and PHINNEY, B. O. (1968) *Physiol. Plant.* **21**, 127.

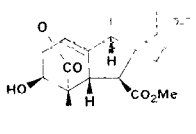
production. The second gene (g_2) apparently blocked a later step (see Scheme 1) since the production of GA_1 and GA_3 only was affected. In the course of our studies[2,3] on the biosynthesis of GAs in mutants of *G. fujikuroi* we fortuitously isolated a mutant R-9 in which the GA pathway is blocked at the same step as the one controlled by the g_2-gene. The mutant R-9 therefore provided a convenient system in which to study the conversion of GA_1 into GA_3.

The conversion of GA_1 into GA_3 in the wild-type strain M-119 of *G. fujikuroi* was investigated by Geissman *et al.*[4,5] who studied the rates of incorporation of *ent*-[17-^{14}C]-kaur-16-en-19-ol and -19-oic acid into GA_4, GA_7, GA_1 and GA_3. After short fermentation times most of the label appeared in GA_4/GA_7 with little in GA_1/GA_3 but after longer fermentation times this distribution was reversed. Although complete separation of the pairs GA_4/GA_7 and GA_1/GA_3 was difficult to achieve, the GA_7/GA_4 and GA_3/GA_1 ratios of radioactivity were found to increase with time and the sequence shown in Scheme 1 was proposed. While our investigations were in progress Pitel *et al*[6] reported a more direct and detailed study of the steps in Scheme 1. They found that no [^{14}C]-GA_1 was converted into [^{14}C]-GA_3 by *G. fujikuroi* strain ACC 917 when grown on a synthetic medium. However, when [1,2-^{3}H]-GA_1 was fed to the same strain grown on a medium favourable to GA_1 production they observed 0·6% conversion to [^{3}H]-GA_3. In both cases GA_3 is produced in high yield and it was therefore concluded that the biosynthesis of GA_3 from GA_4 was mainly via GA_7 and that GA_1 is normally a metabolic end product.

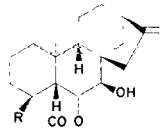
To determine the position of the biosynthetic block in the mutant R-9, the mutant was grown in shake-flask culture on potato-dextrose liquid (PDL) medium[1] for 11 days. The total extract was methylated and examined by GC–MS. The following compounds were identified by comparison of their MS with those of authentic standards: the trimethyl ester (I); GA_{13} trimethyl ester; the aldehydo-dimethyl ester (II); GA_4 methyl ester; GA_7 methyl ester, the methyl ester (III) of the isomeric lactone from GA_7; 7β-hydroxykaurenolide (IV); fujenal (VII); 7β-hydroxy-18-norkaurenolide (V); GA_{17} methyl ester; and 7β,18-dihydroxy-kaurenolide (VI). The isomeric lactone (III) and the 18-norkaurenolide (V) are artefacts respectively formed during GC from GA_7 and 7β,18-dihydroxykaurenolide (VI) (see Ref. 7); the latter compound (VI) is therefore best characterized by GC–MS as the bis-TMSi ether. No trace of GA_1 or GA_3 methyl esters were detected by MS-scanning at their retention times.



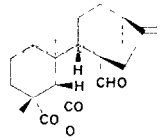
(I) R = CO_2Me
(II) R = CHO



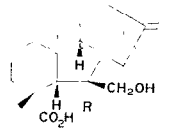
(III)



(IV) R = Me
(V) R = H
(VI) R = CH_2OH



(VII)



(VIII) R = H
(IX) R = T

A more definitive demonstration that the mutant R-9 did not produce GA_1 and GA_3, was provided by growing the mutant on PDL containing [6-^{3}H]-GA_{12}-alcohol (IX), an effective

[2] PHINNEY, B. O. and FUKUJAMI, M. in preparation.
[3] BEARDER, J. R., MACMILLAN, J. and PHINNEY, B O (1974) *Phytochemistry* **13**, to be published.
[4] GEISSMAN, T. A , VERBISCAR, A. J , PHINNEY, B. O and CRAGG, G. (1966) *Phytochemistry* **5**, 933
[5] VERBISCAR, A. J., CRAGG, G., GEISSMAN, T. A. and PHINNEY, B. O. (1967) *Phytochemistry* **6**, 807.
[6] PITEL, D. W., VINING, L. C. and ARSENAULT, G. P A (1971) *Can. J. Biochem.* **49**, 194
[7] CROSS, B. E., GALT, R H. B. and HANSON, J. R. (1963) *J. Chem. Soc.* 3783.

precursor[8] of GA_3 in another mutant (B1-41a) of *G. fujikuroi*. After 11 days the EtOAc extract from the culture filtrates was methylated and subjected to TLC on silica gel with Me_2CO–PE (1:1). There was no radioactivity at the R_f of GA_{12}-alcohol (VIII) and only a trace at the R_f of GA_3 methyl ester where GA_1 and GA_{16} methyl esters would also occur. Most of the activity was located at R_f 0·40–0·65 which was shown to contain labelled GA_4 and GA_7 methyl esters by GC–MS and GC–RC[9] of the trimethylsilylated material recovered from this zone. The GC–RC trace (Fig. 1) showed that the GA_4 derivative (peak 1) had a higher specific activity than the GA_7 derivative (peak 2), suggesting that GA_7 is synthesized faster from its endogenous precursors than from the GA_{12}–alcohol (IX). The absence of significant amounts of labelled GA_3 was further established by the addition of unlabelled GA_3 methyl ester to the methylated extract from the culture filtrates. The radioactivity of GA_3 methyl ester recovered by TLC on silica gel then on alumina was still decreasing rapidly after 4 recrystallizations and corresponded to less than 0·05% incorporation from [6-^{3}H]-GA_{12}-alcohol (IX).

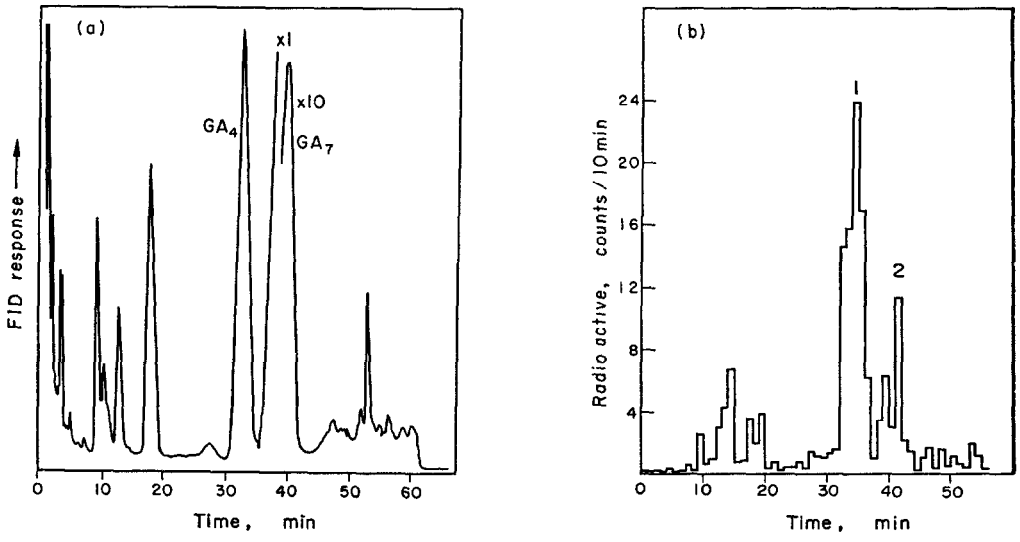


FIG. 1. GA_4- AND GA_7-CONTAINING FRACTION FROM 6-[^{3}H]-GA_{12}-ALCOHOL (IX): (a) GLC AND (b) GC–RC.

To study the metabolism of GA_1 in cultures of the mutant R-9, it was decided to use [1,2-$^3H_2$]-GA_1 despite the uncertainty of the stereochemistry of the tritium labels and therefore of the radiochemical yield in the conversion to [1,2-$^3H_2$]-GA_3. This disadvantage was outweighed by the availability[10] of pure [1,2-$^3H_2$]-GA_1 with high specific activity (5 Ci mmol^{-1}). Very small amounts of the substrate could therefore be used to minimise possible substrate inhibition of the enzyme(s) involved in the conversion to GA_3. The mutant R-9 was cultured on PDL containing [1,2-$^3H_2$]-GA_1 for 11 days. The extract from the culture filtrates, containing 94% of the added radioactivity, was diluted with unlabelled

[8] BEARDER, J. R., MACMILLAN, J. and PHINNEY, B. O. in preparation.

[9] BELHAM, J. E. and NEAL, G. E. (1972) *Anal. Biochem.* **45**, 6.

[10] NADEAU, R. and RAPPAPORT, L. (1972) *Phytochemistry* **11**, 1611. [*Note added in proof.*These authors have recently found the [3H]-GA, of high specific activity (43 Ci mmol^{-1}) contains 9·4% [3H]-GA_3 (13 Ci mmol^{-1}). The [^{3}H]-GA, used here contained no [3H]-GA_3 by partition chromatography on Sephadex G25].

GA_3 and fractionated on a column of Sephadex G25 using the method of Pitel *et al.*[11] The GA_1-fractions contained 90·5% of the radioactivity of the extract. Of the GA_3 containing fractions, the first ten were discarded to avoid possible contamination from tailing of the GA_1 peak. The remaining ten fractions contained 46% of the added GA_3 which was purified by TLC and recrystallized to constant activity. The radioactivity of the recovered GA_3 corresponded to 2·7% of that of the added [1,2-$^3H_2$]-GA_1. Since it has been established[12] that formation of the 1,2-double bond in GA_3 involves the loss of the 1α- and 2α-hydrogen atoms, this result indicates a 2·7% conversion of GA_1 into GA_3. However, the work of Musgrave and Kende[13] suggests that [1,2-$^3H_2$]-GA_1, prepared by catalytic reduction of GA_3 with tritium gas, may contain equal proportions of [1α, 2α- and 1β, 2β-$^3H_2$]-GA_1 since conversion via the 3-tosylate to the 3-ene, GA_5, resulted in the loss of 25% of the [^{3}H]-label. On this basis the conversion of GA_1 to GA_3 would be 5·4%. In the absence of endogenous GA_3, this conversion by the mutant R-9, offers a potential route to GA_3 with high specific radioactivity. However, no significant conversion of GA_1 into GA_3 was observed when the mutant R-9 was cultured in the presence of larger quantities of unlabelled GA_1.

EXPERIMENTAL

Provenance of Gibberella fujikuroi *Mutant R-9.* The mutant R-9 was obtained from a wild-type strain N-3844 of *G. fujikuroi*, isolated in the field in Japan. During subculturing of this strain perithecia appeared fortuitously. The ascospores were collected unordered from these perithecia; they were germinated on agar then transferred to agar slopes as 500 different strains of which the mutant R-9 was one. The mutant R-9 is therefore homocaryotic in origin.

GC–MS. A GEC-AEI MS30 dual beam mass spectrometer was used coupled to a Pye-Unicam 104 gas chromatogram via a silicone membrane separator Silanized glass columns (152 4 × 0·32 cm) were packed with de-mineralized and silanized Gaschrom A coated with 2% QF1. The He-flow rate was 30 ml min.$^{-1}$ The MS were determined at 24 eV at a source temp. of 210° and a separator temp. of 185° with a scan speed of 6·5 sec per mass decade. The spectra were recorded with an on-line DEC Linc 8 computer

GC–RC. The effluent from the F I D. of a Pye-Unicam 104 gas chromatogram was collected as described by Belham and Neal.[9] Fractions were collected at 1 min intervals and counted as described below.

Radioactivity determination. An ICN Tracerlab Corumatic 200 was used for scintillation counting. Samples in toluene or MeOH were counted in toluene (10 ml) containing butyl PBD (5 g l^{-1}) with an efficiency of 56%. Samples dissolved in H_2O were counted in toluene–2-methoxyethanol (3·2) containing butyl PDP (5 g l^{-1}) with an efficiency of 32%.

Conditions of culture. The mutant R-9 was grown in shake-culture on a potato-dextrose liquid (PDL) medium.[1,5] After 3 transfers to optimize homogeneity, the cultures were grown for 11 days in the absence or presence of substrates which were added in MeOH to a shake flask containing PDL (100 ml) medium immediately after autoclaving

Analysis of metabolites. (1) *Without added substrate.* The culture (100 ml) was centrifuged at 4000 rpm for 15 min; the supernatant was removed and the mycelium was re-suspended in H_2O and re-centrifuged. The combined supernatant fractions were then extracted with EtOAc (3 × 100 ml) at pH 2·5. The material recovered from the EtOAc was methylated and examined by GC–MS with GC temp. programming from 215° at 2° min^{-1}. The following compounds were identified from their MS (in order of increasing retention time): (a) Me_3 *ent*-6,7-*seco*kaur-16-en-6,7,19-trioate (I) with *m/e* (% base peak) 406 (M^+, 1), 375 (2), 315 (2), 255 (3), 227 (38), 195 (100), 167 (52) and 101 (68); (b) Me_3GA_{13} with MS identical to the published MS,[14] (c) Me_2 *ent*-7-oxo-6,7-*seco*kaur-16-en-6,19-dioate (II) with *m/e* (%) 376 (M^+, 1), 345 (2), 307 (2), 227 (25), 195 (90), 167 (56), 135 (9), 109 (22), 107 (100), 105 (5), 93 (6), 91 (6), 81 (4) and 79(6); (d) $MeGA_4$ identical to the published spectrum;[14] (e) $MeGA_7$,[14] (f) the isomeric lactone (III) of $MeGA_7$ with MS virtually identical to that of $MeGA_7$,[14] (g) *ent*-6β,7α-dihydroxykaur-16-en-19-oic acid 19,6-lactone (IV) with *m/e* (%) 316 (M^+, 1), 314(2), 298(30), 283(8), 270(13), 255(11), 227(10), 205(7), 137(100) and 109 (98); (h) the 6,19-anhydride (VII) with *m/e* (%) 330 (M^+, 11), 312 (1), 302 (1), 284 (3), 181 (11), 153 (50),

[11] PITEL, D. W., VINING, L. C. and ARSENAULT, G. P. A. (1971) *Can. J. Biochem.* **49,** 185.
[12] EVANS, R , HANSON, J. R. and WHITE, A. F. (1970) *J. Chem. Soc.* **C,** 2601.
[13] MUSGRAVE, A and KENDE, H. (1970) *Plant Physiol.* **45,** 56.
[14] BINKS, R , MACMILLAN, J. and PRYCE, R. J. (1969) *Phytochemistry* **8,** 271.

150 (20), 135 (9), 131 (8), 121 (32), 109 (100), 93 (26), 91 (11), 81 (9), 79 (25), 67 (11), 55 (7) and 43 (23); (i) *ent*-6β,7α-dihydroxy-18-norkaur-16-en-19- oic acid 19,6-lactone (V) with *m/e* (%) 302 (M^+, 3), 284 (62), 269 (23), 256 (10), 241 (18), 191 (27), 131 (39), 123 (33), 121 (30), 119 (28), 107 (30), 105 (25), 95 (100), 93 (52) and 91 (30); and (j) *ent*-6β,7α,18-trihydroxykaur-16-en-19-oic acid 19,6-lactone (VI), the bis TMSi ether had *m/e* (%) 476 (M^+, 1), 461 (4), 446 (1), 433 (3), 286 (35), 358 (8), 343 (8), 296 (22), 283 (52), 268 (100), 103 (22), 75 (25) and 73 (70). (2) *With added substrate*, [6-^{3}H]-GA_{12} alcohol (IX). A culture (100 ml) was grown on PDL to which [6-^{3}H]-GA_{12} alcohol (*ca.* 550 μg, $2{\cdot}56 \times 10^6$ decomp. min^{-1}) had been added in MeOH (350 μl). After 11 days, the culture filtrate was worked up as in (1) to give a total extract (25·5 mg, $2{\cdot}35 \times 10^6$ decomp. min^{-1}, 91% recovery) which was methylated with CH_2N_2. A portion (*ca.* $1{\cdot}5 \times 10^5$ decomp. min^{-1}) of the methylated extract was examined by TLC on 2 silica gel G plates (20 × 5 cm), developed with Me_2CO–PE (1:1). A marker plate with the Me esters of GA_3, GA_{16}, GA_4, GA_7, GA_{14}, GA_{13} and GA_{12} alcohol was developed under the same conditions. One of the plates bearing the extract was divided into 30 strips (0·5 cm) and the silica gel from each strip was scraped off into vials containing scintillation fluid. The vials were shaken vigorously then allowed to settle for 10 min before being counted for radioactivity. The zone at R_f 0·40–0·65 corresponding to the R_f values of $MeGA_4$ and $MeGA_7$ on the marker plate contained most of the radioactivity. The same zone from the second TLC plate bearing the extract was removed and extracted with EtOAc The recovered material in pyridine was treated with Me_3SiCl and $(Me_3Si)_2NH$ then examined by GC–MS on a 2% QF1 column at 215°. The two major peaks had MS identical with the published spectra of the Me ester TMSi ether derivatives of GA_4 and GA_7. GC–RC (Fig. 1) showed that these two peaks were radioactive. Another portion ($9{\cdot}03 \times 10^5$ decomp. min^{-1}) of the methylated total extract from the culture was diluted with unlabelled Me GA_3 (30·0 mg) and subjected to TLC on silica gel developed (×3) with Et_2O–PE (5:1). The material, recovered from R_f 0·3 in EtOAc, was re-chromatographed on a layer of Al_2O_3 developed with EtOAc. The Me GA_3 (7100 decomp.) recovered from R_f 0·3 in EtOAc, was recrystallized (×4) from EtOAc–PE with the following decomp. min^{-1} mg^{-1}: 198, 55, 41 and 15. (3) *With added substrate*, [1,2-$^3H_2$]-GA_1. [1,2-$^3H_2$]-GA_1 ($7{\cdot}04 \times 10^5$ decomp. min^{-1}, 22 μg) in MeOH (200 μl) was added as described in (2). The culture (100 ml) was grown and worked up as described in (1) to give a crude extract ($6{\cdot}62 \times 10^5$ decomp. min^{-1}, 94% recovery) to which unlabelled GA_3 (31·5 mg) was added. A Sephadex G25 column (80 × 1·5 cm) was made up using the solvent system, C_6H_6–EtOAc–AcOH–H_2O (11:5:6:10). The extract ($6{\cdot}11 \times 10^5$ decomp. min^{-1}) was dissolved in the minimum vol. of the aqueous phase, absorbed into Sephadex G25 (200 mg) which was placed on top of the column. The column was then eluted with the organic phase and fractions (10 ml) were monitored for radioactivity by taking aliquots (250 μl) from alternate fractions. The [1,2-$^3H_2$]-GA_1 was eluted in fractions 65–82 which contained all the significant radioactivity ($5{\cdot}53 \times 10^5$ decomp. min^{-1}, 90·5% recovery). The subsequent fractions were monitored for GA_3 by spotting on silica gel G layers which were then sprayed with 5% H_2SO_4 in EtOH. Gibberellin A_3 was detected (blue fluorescence in UV at 365 nm after heating at 120° for 10 min) in fractions 84–104. Fractions 94–104 were bulked to give GA_3 as a crystalline solid (14 mg, 704 decomp. min^{-1} mg^{-1}). TLC on silica gel G in EtOAc–$CHCl_3$–AcOH (4:5:1) removed a yellow contaminant. The material at R_f 0·4 was recovered to give GA_3 which was recrystallized from EtOAc–PE to constant activity: 1st crystallization, 580; 2nd recrystallization, 581; 3rd recrystallization 576 decomp. min^{-1} mg^{-1}. (4) *With added substrate, unlabelled* GA_1. A culture (100 ml) was grown on GA_1 (500 μg) and worked up as in (II). The crude extract from the culture filtrate was methylated then trimethylsilylated. GC on 2% QF1 at 210° showed that the GA_1 had been unmetabolized and no detectable quantity of GA_3 had been formed.

Partial synthesis of gibberellin A_{12}-alcohol (VIII). Gibberellin A_{12}-aldehyde[3] (40 mg) in EtOH (20 ml) was treated with $NaBH_4$ (80 mg) at 0°. After 5 hr the solution was worked up as usual to give a semi-crystalline foam which was purified by TLC on silica gel G in EtOAc–PE–AcOH (50:50:1). Elution of the band at R_f 0·3 gave *GA_{12}-alcohol* (26 mg) m.p. 190–193° (from EtOAc–PE) (Found: M^+ 318·219. $C_{20}H_{30}O_3$ requires: M 318·219); *m/e* (% base peak) 318 (17), 300 (94), 286 (47), 273 (25), 256 (56), 242 (31), 240 (28), 105 (67) and 91 (100); ν_{max} (Nujol) 3310, 1697, 1655 and 879 cm^{-1}; τ-values (C_5D_5N) 9·14 (3H, *s*, 20-H), 8·58 (1H, *d*, *J* 12 Hz, 5-H), 8·43 (3H, *s*, 18-H), 7·08 (1H, *dd*, *J* 12, 6 Hz, 6-H), 5·94 and 5·65 (each 1H, ABM, *J* 10, 6 Hz, 7-H_2, 5·07 (1H, *br*, 17-H) and 4·97 (1H, *br*, 17-H). The Me ester had *m/e* (% base peak) 332 (M^+, 25), 314 (75), 300 (25), 299 (31), 255 (100), and 91 (66). The Me TMSi ether had *m/e* (% base peak) 404 (M^+, 17), 389 (28), 372 (27), 344 (11), 314 (70), 299 (27), 255 (100), 254 (79), 241 (69), 239 (60) and 185 (70).

Partial synthesis of [6-^{3}H]-*GA_{12}-alcohol* (IX). The above reaction was carried out using [6-^{3}H]-GA_{12}-aldehyde[3] and the reaction mixture was purified by liquid–liquid partition chromatography as described[3] for the separation of [6-^{3}H]-GA_{12} and -GA_{12}-aldehyde. The [6-^{3}H]-GA_{12}-alcohol (IX) was eluted in fractions 21–28 and crystallized to constant activity (4 mg, 5320 decomp. min^{-1} mg^{-1}).

Acknowledgements—We thank the S.R.C. and Tate & Lyle Ltd. for an S.R C. CAPS Studentship (J R.B.), the S.R.C. for a Research Grant to purchase the AEI-GEC MS30 mass spectrometer, and Professor L. Rappaport for a generous sample of 1,2-[^{3}H]-GA_1. We also thank Professor L. C. Vining for disclosing details of Sephadex partition chromatography prior to publication.