

Biosynthetic Origin of Gibberellins A₃ and A₇ in Cell-Free Preparations from Seeds of *Marah macrocarpus* and *Malus domestica*¹

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ABSTRACT

Cell-free preparations from seeds of *Marah macrocarpus* L. and *Malus domestica* L. catalyzed the conversion of gibberellin A₉ (GA₉) and 2,3-dehydroGA₉ to GA₇; GA₉ was also metabolized to GA₄ in a branch pathway. The preparation from *Marah* seeds also metabolized GA₅ to GA₃ in high yield; GA₄ was a minor product and was not metabolized to GA₃. Using substrates stereospecifically labeled with deuterium, it was shown that the metabolism of GA₅ to GA₃ and of 2,3-dehydroGA₉ to GA₇ occurs with the loss of the 1β-hydrogen. In cultures of *Gibberella fujikuroi*, mutant B1-41a, [1β,2β-²H₂]GA₄, was metabolized to [1,2-²H₂]GA₃ with the loss of the 1α- and 2α-hydrogens. These results provide further evidence that the biosynthetic origin of GA₃ and GA₇ in higher plants is different from that in the fungus *Gibberella fujikuroi*.

In cultures of the fungus *Gibberella fujikuroi*, GA₄ (Fig. 1)² is first metabolized (6) to GA₇ (Fig. 1, [4]) and then to GA₃ (Fig. 1, [5]). The stereochemistry of the didehydrogenation of GA₄ (Fig. 1, [1]) to GA₇ in this fungus was first investigated by Evans *et al.* (12). These workers concluded from incorporation of tritium from 2R-, 2S-, and 5R-³H-labeled [2-¹⁴C]-mevalonic acids that the 1α- and 2α-hydrogens of GA₄ were lost. More recently (8), using the mutant B1-41a of *G. fujikuroi* in which GA biosynthesis is genetically blocked at a step before GA₄, Beale and MacMillan (3) showed that [1β-²H₁]-GA₄ (Fig. 1, [2]) (22) was metabolized to GA₃ with retention of the deuterium label. Using the same mutant, the present paper reports the metabolism of [1β,2β-²H₂]GA₄ (Fig. 1, [3]) (23) into GA₃ with retention of both deuterium labels. Thus, it has been firmly established that GA₄ is converted into GA₇ by *cis*-1α,2α-didehydrogenation in *G. fujikuroi*. To date, no investigation of the stereochemistry of this conversion has been reported for higher plants. However, in contrast to the fungus in which 1α- and 2α-hydroxylated GAs predominate, higher plants contain predominantly 1β- and 2β-hydroxylated GAs (see Bearder [5] for review). This suggested the possibility that the stereochemistry of the formation of the 1,2-double

bond in GA₇ and GA₃ in higher plants was opposite to that in the fungus and involved the loss of the 1β- and 2β-hydrogens.

When our investigation of this possibility began, the natural occurrence of GA₃ in higher plants had not been conclusively established. However, GA₇ had been identified by GC-MS in extracts of seeds of *Marah macrocarpus* L. (9) and *Malus domestica* L. (19, 27). Accordingly, cell-free preparations from the seed of these two species were used to investigate the possible formation of GA₇ from GA₄ and the stereochemistry of the process. Attempts over many seasons failed to provide enzyme preparations that metabolized GA₄ to GA₇. The recent findings of Fujioka *et al.* (14, 15) established the natural occurrence of GA₃ in shoots of *Zea mays* L. and the biosynthesis of GA₃ from GA₂₀ (Fig. 1, [6]) via GA₅ (Fig. 1, [8]) (15). These results from *Zea mays* suggested that GA₇ in *M. macrocarpus* and *M. domestica* may not be formed by direct 1,2-didehydrogenation of GA₄ but from GA₉ via 2,3-dehydroGA₉ (Fig. 1, [9]). This paper presents evidence for the enzymatic formation of GA₇ from 2,3-dehydroGA₉ by cell-free enzyme preparations from *Marah* and *Malus* seeds and of GA₃ from GA₅ in the enzyme preparation from *Marah* seeds. Investigations on the stereochemistry of these transformations are also reported.

MATERIALS AND METHODS

General Procedures

Solvents were redistilled before use. TLC analysis was performed on aluminum sheets, coated with 2 mm layers of silica gel 60 F-254 (Merck, Darmstadt, FRG), and flash chromatography (29) was performed with Kieselgel 60 (40–63 μm) (Merck, Darmstadt, FRG). NMR chemical shifts were determined relative to internal tetramethylsilane. GC, GC-MS, and GC-SIM³ analyses were performed on capillary glass wall-coated OV-1 columns (GC² [Chromatography], Northwich, Cheshire, U.K.) for samples that were methylated using ethereal diazomethane then trimethylsilylated using *N*-

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² The numbers for chemical structures are given in square brackets.

³ Abbreviations: GC-SIM, combined gas chromatography-selected ion monitoring; EtOAc, ethyl acetate; AMO 1618, 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methylchloride; MeTMSi, methyl ester trimethylsilyl ether; PE, light petroleum ether b.p. 60–80°C; THF, tetrahydrofuran; KRI, Kovats retention index; Rt, retention time.

methyl-*N*-trimethylsilyltrifluoroacetamide (Pierce and Warriner, Chester, UK). The instrumentation and conditions used for GC-MS and GC-SIM analyses have been described by Gaskin *et al.* (16) and Fujioka *et al.* (13), respectively. In the descriptions of the preparation of labeled substrates the standard work-up procedure comprised the addition of water, acidification with 2 M hydrochloric acid to pH 3, and recovery of organic material in an EtOAc extract.

Conversion of [$1\beta,2\beta\text{-}^2\text{H}_2$] Gibberellin A₄ to [$1,2\text{-}^2\text{H}_2$] Gibberellin A₃ by Cultures of *Gibberella fujikuroi*, mutant B1-41a

A conical flask containing a 40 mL solution of 40% ICI medium, as defined by Geissman *et al.* (17), and 2 mg AMO 1618 was inoculated with a 1 mL mycelium suspension of *Gibberella fujikuroi*, mutant B1-41a. The culture was grown at 25°C on an orbital shaker at 2100 rpm for 5 d. The mycelium was collected by filtration under sterile conditions and resuspended in 50 mL 0% ICI medium containing 2 mg AMO 1618 and 1 mg [$1\beta,2\beta\text{-}^2\text{H}_2$]GA₄ (Fig. 1) (23) with 1.65 atoms deuterium per molecule. The culture was grown for 6 d at 25°C on an orbital shaker and filtered from the mycelial mat, which was washed with water. The combined filtrate and washings were acidified with 2 M hydrochloric acid and extracted with EtOAc. The organic phase was extracted with aqueous sodium hydrogen carbonate solution. The aqueous phase was acidified with 2 M hydrochloric acid and extracted with EtOAc. The EtOAc extract was washed with water, dried over anhydrous Na₂SO₄, and evaporated to give the crude product, which was methylated and trimethylsilylated for GC-MS. The metabolites were identified as the MeTMSi derivatives by full scan MS and the deuterium content determined by GC-SIM on the M⁺ ion cluster.

Preparation of Cell-Free Extracts

Seeds of *Marah macrocarpus* L. were collected from Latigo Canyon, CA in April 1987 and stored at -196°C. Seeds in which the cotyledons were half the length of the seed were thawed and dissected to provide cotyledons and endosperm (40 g total) minus the testae. The cotyledon/endosperm mixture was pulverized at -196°C and homogenized at 4°C in 20 mL 0.1 M Tris HCl buffer containing 0.25 M sucrose and 2 mM DTE. The homogenate was centrifuged at 36,000g for 1 h at 4°C. To the supernatant was added a solution of ammonium sulfate in 0.25 M Tris HCl buffer (pH 7.6) to give a final concentration of 70% (w/v). After stirring for 1 h at 4°C, the precipitated protein was centrifuged at 36,000g for 1 h at 4°C. The pelleted protein was washed with 70% (w/v) aqueous ammonium sulfate and centrifuged at 36,000g. The pellets were dissolved in 3 mL 0.1 M Tris HCl buffer (pH 7.6) and divided into aliquots that were stored at -196°C for incubations with labeled substrates.

Seeds of *Malus domestica* L., collected from fruit 10 weeks after anthesis, contained no liquid endosperm. The cotyledons (2.5 g) were collected, pulverized in liquid N₂, and homogenized in 5 mL Tris HCl buffer, and cell-free extracts were prepared as for *Marah* seeds.

Incubations with Labeled Substrates

Each substrate (5 μg) was incubated for 2 h at 30°C in 0.5 mL of a cell-free extract containing 1 mM Fe²⁺, 10 mM ascorbate, and 10 mM 2-oxoglutarate. The incubation mixture was acidified to pH 3.0 with 2 M hydrochloric acid and extracted with EtOAc (1 mL × 3). The EtOAc extract was reduced to near dryness with a stream of N₂. The residue was dissolved in 0.5 mL 80% (v/v) aqueous methanol and partitioned twice against PE. The PE extract was discarded and the aqueous methanol solution was evaporated to near dryness with a stream of N₂. The residue, dissolved in methanol, was methylated with diazomethane in ether and the solution was evaporated. The residue was dissolved in EtOAc (1 mL), and the solution was extracted twice with water to remove carbohydrates. The EtOAc was removed with a stream of N₂, and the residual methylated sample was trimethylsilylated for GC-MS analysis.

Provenance of Labeled Substrates

The following labeled substrates were used: (a) [$17\text{-}^{13}\text{C},^3\text{H}_2$]-GA₅, prepared by Fujioka *et al.* (13); (b) [$17\text{-}^{13}\text{C},^3\text{H}_2$]GA₂₀, prepared by Ingram *et al.* (20); (c) [$17\text{-}^{13}\text{C},^3\text{H}_2$]GA₄, prepared by Dr. M. H. Beale (unpublished); (d) [$17\text{-}^2\text{H}_2$]GA₉; (e) [$17\text{-}^2\text{H}_2$]-2,3-dehydroGA₉; (f) [$1\alpha\text{-}^2\text{H}_1$]GA₅; (g) [$1\beta,3\text{-}^2\text{H}_2$]GA₅; and (h) [$1\beta,3\text{-}^2\text{H}_2$]-2,3-dehydroGA₉. The preparation of substrates (d) to (h) is described in the following paragraphs.

[$17\text{-}^2\text{H}_2$]Gibberellin A₉

The known (10) GA₉ methyl ester norketone (Fig. 2, [11]) (50 mg) in 5 mL dry THF was stirred in an atmosphere of N₂

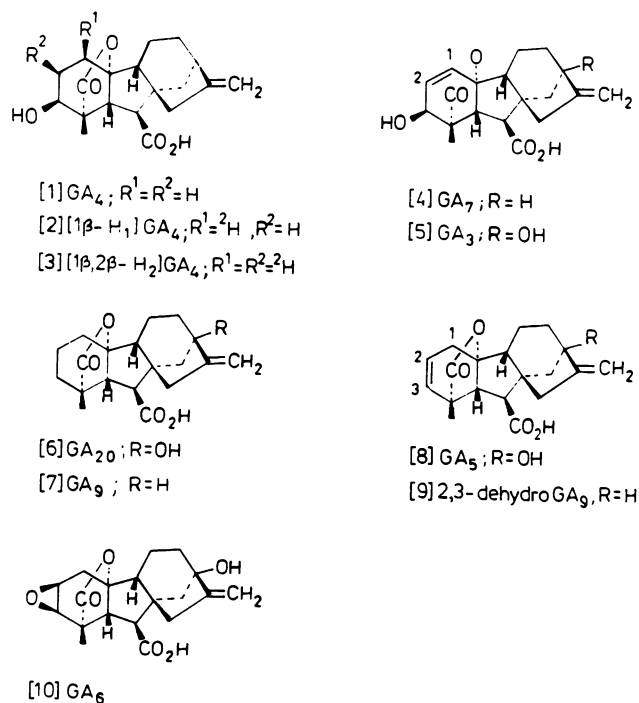


Figure 1. GA structures referred to in the text.

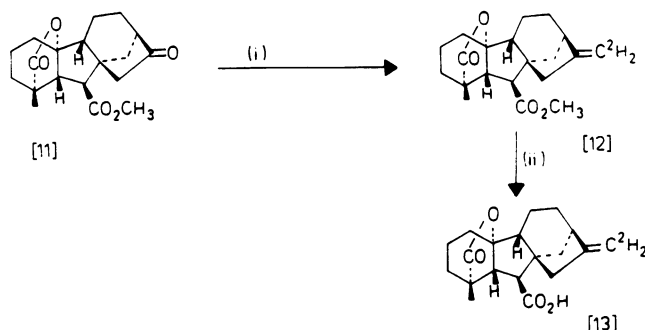


Figure 2. Synthesis of $[17\text{-}^2\text{H}_2]\text{GA}_9$. Reagents: (i) Zn, $\text{C}^2\text{H}_2\text{Br}_2$, TiCl_4 , THF; (ii) 10 M NaOH.

gas at room temperature for 1 h with 5 mL of methylenating reagent, prepared (21) from 1.14 g zinc powder, $\text{C}^2\text{H}_2\text{Br}_2$ (0.4 mL, 99.2% deuterium), 0.4 mL titanium tetrachloride, and 10 mL dry THF. The standard work-up gave a crude product, which was purified by flash chromatography. Elution of the column with 10 to 20% (v/v) EtOAc in PE gave 23 mg $[17\text{-}^2\text{H}_2]\text{GA}_9$ methyl ester (Fig. 2, [12]): $^1\text{H-NMR}$ (CDCl_3), δ -values (assignments) 1.08(s, 18- H_3), 2.54(d, $J = 11$ Hz, 5-H), 2.71(d, $J = 10$ Hz, 6-H), and 3.70(s, OCH_3); MS, m/z values (relative intensities) 332 (M^+ , 3%), 300(70), 288(10), 272(100), 243(67), 228(70), 183(11), and 157(28).

A 12 mg portion of the methyl ester in 5 mL 10 M NaOH was heated under reflux for 16 h. The standard work-up, followed by flash chromatography and elution with EtOAc:PE:CH₃CO₂H (50:50:0.1, v/v/v), gave 4 mg $[17\text{-}^2\text{H}_2]\text{GA}_9$ (Fig. 2, [13]) containing 1.70 deuterium atoms per molecule: $^1\text{H-NMR}$ (CDCl_3), δ -values (assignments) 1.13(s, 18- H_3), 2.50(d, $J = 10$ Hz, 5-H), and 2.74(d, $J = 10$ Hz, 6-H).

$[17\text{-}^2\text{H}_2]2,3\text{-Dehydrogibberellin A}_9$

A mixture (6 g) of GA_4 (Fig. 1) (70%) and GA_7 (Fig. 1) (30%) was methylated with ethereal diazomethane, and the mixture of methyl esters in THF:water (1:1, v/v) was stirred at 4°C with 9.6 g sodium periodate and one crystal of osmium tetroxide. The mixture was allowed to reach room temperature and stirring was continued for 16 h. The standard work-up followed by crystallization from acetone-PE gave 4.0 g of the known (18) GA_4 methyl ester norketone (Fig. 3, [14]), m.p. 194 to 196°C. A 300 mg portion of the norketone in 15 mL dry pyridine was heated under reflux with 0.5 mL phosphoryl chloride for 30 min. The standard work-up, followed by flash chromatography and elution with 18% (v/v) EtOAc in PE, gave 214 mg of the known (10) 2,3-dehydro GA_9 methyl ester norketone (Fig. 3, [15]): $^1\text{H-NMR}$ (CDCl_3), δ -values (assignments) 1.24(s, 18- H_3), 2.69(d, $J = 10$ Hz, 5-H), 2.82(d, $J = 10$ Hz, 6-H), 3.74(s, OCH_3), 5.68(dt, $J = 3$ and 10 Hz, 3-H), and 5.83(dt, $J = 3$ and 10 Hz, 2-H). A 30 mg portion of the ketone in 5 mL of dry THF was stirred with 5 mL of the methylenating agent, prepared (21) from 1.14 g zinc powder, 0.4 mL $\text{C}^2\text{H}_2\text{Br}_2$ (99.2% deuterium), 0.4 mL titanium tetrachloride, and 10 mL of dry THF. The standard work-up,

followed by flash chromatography and elution with 10-20% (v/v) EtOAc in PE, yielded 20 mg of $[17\text{-}^2\text{H}_2]2,3\text{-dehydroGA}_9$ methyl ester (Fig. 3, [16]) containing 1.70 deuterium atoms per molecule: $^1\text{H-NMR}$ (CDCl_3), δ -values (assignments) 1.22(s, 18- H_3), 2.66(d, $J = 10$ Hz, 5-H), 2.79(d, $J = 10$ Hz, 6-H), 3.71(s, CO_2CH_3), 5.67(dt, $J = 3$ and 10 Hz, 3-H), and 5.82(dt, $J = 3$ and 10 Hz, 2-H). This methyl ester (19 mg) in 5 mL of 10 M NaOH was heated under reflux for 16 h. The standard work-up gave a gum that was treated with 3 M hydrochloric acid in methanol (pH 1-2) for 1 h (to relaxonize). The standard work-up, followed by flash chromatography and elution with EtOAc:PE:CH₃CO₂H (50:50:0.1, v/v/v), gave 11 mg of $[17\text{-}^2\text{H}_2]2,3\text{-dehydroGA}_9$ (Fig. 3, [17]) containing 1.67 deuterium atoms per molecule. $^1\text{H-NMR}$ (CDCl_3), δ -values (assignments) 1.24(s, 18- H_3), 2.66(d, $J = 10$ Hz, 5-H), 2.76(d, $J = 10$ Hz, 6-H), 5.67(dt, $J = 3$ and 10 Hz, 3-H), and 5.81(dt, $J = 3$ and 10 Hz, 2-H); MS, m/z values (relative intensities) (as methyl ester) 299(5%), 296(42), 257(5), 226(100), 211(38), 181(54), 156(51), 143(19), 105(30), and 85(25).

$[1\alpha\text{-}^2\text{H}_1]\text{Gibberellin A}_5$

The known (22) GA_3 methyl ester 13-acetate (4.2 g) was converted into 5.1 g of the 3-methanesulfonyl derivative (Fig. 4, [18]), m.p. 110-113°C, as described (24) for the $[2\text{-}^2\text{H}]\text{-labeled GA}_3$ methyl ester 13-acetate. The methanesulfonate (200 mg) was hydrogenolyzed by the method of MacMillan and Willis (24). Palladium on calcium carbonate (10%, w/w; 100 mg) and 0.5 mL cyclohexene in 5 mL EtOAc were stirred for 30 min in an atmosphere of deuterium gas. $[^2\text{H}_5]\text{Pyridine}$ (0.5 mL) was then added to poison the catalyst. After 10 min the methanesulfonate (200 mg) was added in 2 mL EtOAc, and the mixture was stirred for 2 h at room temperature. The mixture was then filtered and the filtrate was evaporated under vacuum to give a gum that was fractionated by flash chromatography eluting with 15 mL portions of 15 to 25% (v/v) EtOAc in PE. Fraction 13 contained 17 mg $[1\alpha\text{-}^2\text{H}_1]\text{GA}_5$ methyl ester 13-acetate (Fig. 4, [19]). Fractions 14 to 16 contained 47 mg of a 1:1 mixture of $[1\alpha\text{-}^2\text{H}_1]\text{GA}_5$ methyl ester 13-acetate and the isomeric compound (Fig. 4, [20]). This mixture (45 mg), 2 mL of acetic anhydride, and 5 mg of

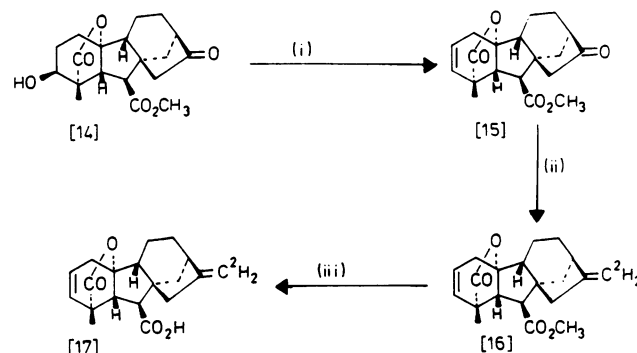


Figure 3. Synthesis of $[17\text{-}^2\text{H}_2]2,3\text{-dehydroGA}_9$. Reagents: (i) POCl_3 , pyridine; (ii) Zn, $\text{C}^2\text{H}_2\text{Br}_2$, TiCl_4 , THF; (iii) 10 M NaOH.

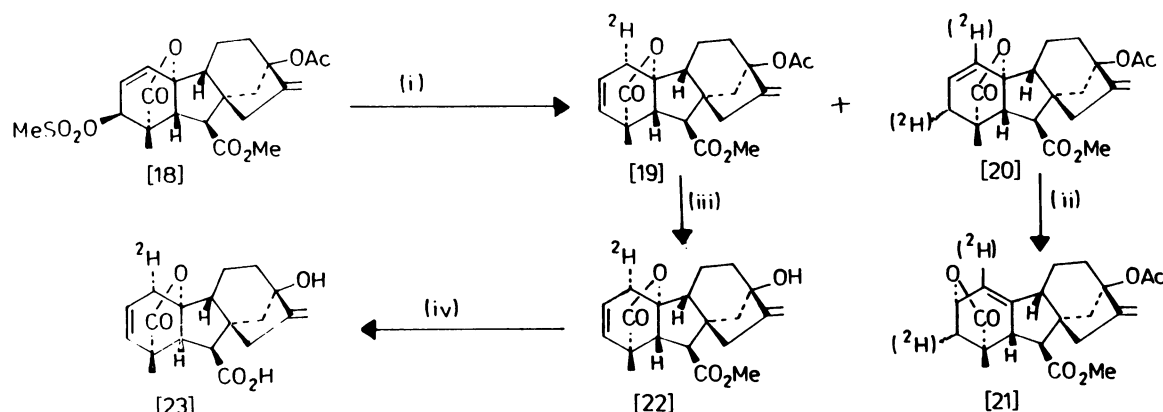


Figure 4. Preparation of $[1\alpha\text{-}^2\text{H}_1]\text{GA}_5$. Reagents: (i) D_2 , 10% Pd on CaCO_3 , cyclohexene, $\text{C}_5\text{D}_5\text{N}$, EtOAc; (ii) $p\text{-MeC}_6\text{H}_4\text{SO}_3\text{H}$, $(\text{MeCO})_2\text{O}$; (iii) K_2CO_3 , MeOH; (iv) 10 M NaOH.

toluene-4-sulfonic acid were stirred at room temperature for 4 h. The standard work-up and flash chromatography, eluting with 18% (v/v) EtOAc in PE, gave 26 mg $[1\alpha\text{-}^2\text{H}_1]\text{GA}_5$ methyl ester 13-acetate containing 0.55 deuterium atoms per molecule: ^2H -NMR (CHCl_3), δ -value (assignment) 2.56($1\alpha\text{-}^2\text{H}_1$); ^1H -NMR (CDCl_3), δ -values (assignments) 1.22(s, 18- H_3), 2.02(s, 13- OCOCH_3), 2.66(d, $J = 10$ Hz, 5-H), 2.80(d, $J = 10$ Hz, 6-H), 3.72(s, OCH_3), 4.98 and 5.12 (br, 17- H_2), 5.67(dd, $J = 2.5$ and 10.5 Hz, 3-H), and 5.80(dd, $J = 2.5$ and 10.5 Hz, 2-H); MS, m/z values (relative intensities) 387(M^+ , 21%), 356(12), 345(25), 301(10), 283(100), 268(9), 240(9), 222(21), 208(17), and 194(7). Next, 20 mg of $[1\alpha\text{-}^2\text{H}_1]\text{GA}_5$ methyl ester 13-acetate in 5 mL methanol was stirred for 16 h with 5

mL aqueous potassium carbonate (saturated solution diluted $\times 20$). The standard work-up, followed by crystallization from EtOAc-PE, gave 13.5 mg $[1\alpha\text{-}^2\text{H}_1]\text{GA}_5$ methyl ester (Fig. 4, [22]), containing 0.53 deuterium atoms per molecule; ^2H -NMR (CHCl_3), δ -value (assignment) 2.57($1\alpha\text{-}^2\text{H}_1$); ^1H -NMR (CDCl_3), δ -values (assignments) 1.23(s, 18- H_3), 2.66(d, $J = 10$ Hz, 5-H), 2.79(d, $J = 10$ Hz, 6-H), 3.73(s, OCH_3), 4.96 and 5.25(each br, 17- H_2), 5.67(dd, $J = 2$ and 11.5 Hz, 3-H) and 5.80(dd, $J = 2$ and 11.5 Hz, 2-H); MS, m/z values (relative intensities) 345(M^+ , 22%), 242(38), 241(100), 168(15), 158(28), 157(47), 137(30), and 106(15). Finally, 13 mg $[1\alpha\text{-}^2\text{H}_1]\text{GA}_5$ methyl ester in 5 mL 10 M NaOH was heated under reflux for 16 h. The standard work-up, followed by flash

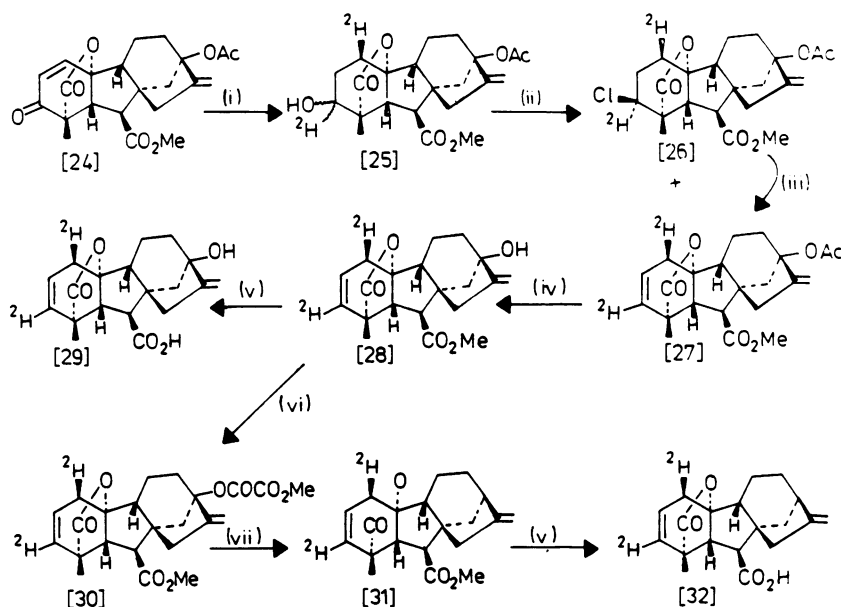
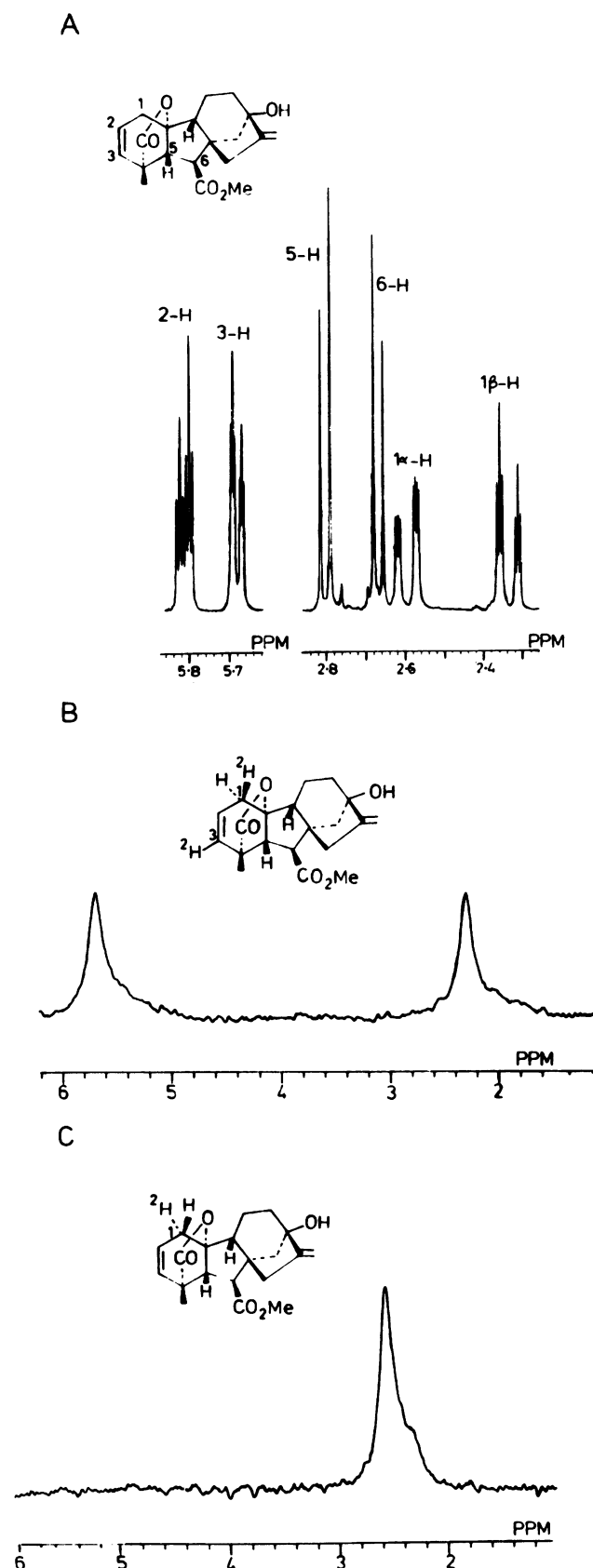


Figure 5. Preparation of $[1\beta,3\text{-}^2\text{H}_2]\text{GA}_5$ and $[1\beta,3\text{-}^2\text{H}_2]\text{-2,3-dehydroGA}_9$. Reagents: (i) $\text{Na-B}^2\text{H}_4$, Cu(I)Cl , MeOH; (ii) POCl_3 , pyridine; (iii) DBU, pyridine; (iv) K_2CO_3 , MeOH- H_2O (5:1); (v) 10 M NaOH; (vi) $\text{MeO}_2\text{C}\cdot\text{COCl}$, THF, pyridine; (vii) $^t\text{Bu}_3\text{SnH}$, AIBN, $\text{C}_6\text{H}_5\text{Me}$.



chromatography and elution with 50 to 60% (v/v) EtOAc in PE containing 0.1% (v/v) $\text{CH}_3\text{CO}_2\text{H}$, gave 2.8 mg $[1\alpha\text{-}^2\text{H}_1]\text{-GA}_5$ (Fig. 4, [23]) containing 0.50 deuterium atoms per molecule; $^1\text{H-NMR}$ (CD_3COCD_3), δ -values (assignments) 1.19(s, 18- H_3), 2.55(d, $J = 10$ Hz, 5-H), 2.75(d, $J = 10$ Hz, 6-H), 4.86 and 5.19(each br, 17- H_2), 5.70(dd, $J = 2.5$ and 9 Hz, 3-H), and 5.85(dd, $J = 2.5$ and 9 Hz, 2-H); MS, m/z values (relative intensities) 331(M^+ , 29%), 286(100), 242(33), 240(22), 168(13), 158(29), 157(33), and 106(41).

$[1\beta,3\text{-}^2\text{H}_2]$ Gibberellin A_5

Copper (I) chloride (750 mg) was added to 1.8 g of the known (4) 3-oxo GA_3 methyl ester 13-acetate (Fig. 5, [24]) in 100 mL methanol with stirring at room temperature. After 5 min, 320 mg NaB^2H_4 was added to the reaction mixture. After 1 h, the original black color had changed to green and the solvent was removed under vacuum. The standard work-up gave a crude product, which was purified by flash chromatography. Elution with 30 to 60% (v/v) EtOAc in PE gave a 1.06 g mixture of the 3-epimeric alcohols (Fig. 5, [25]), in the ratio 85:15 of the $3\alpha:3\beta$ -alcohols (determined by $^1\text{H-NMR}$). This mixture (1.0 g) in 50 mL dry pyridine and 1.8 mL phosphoryl chloride was heated under reflux for 45 min. Water was added slowly to the cooled solution, followed by the standard work-up. The crude product was subjected to flash chromatography, and elution with 18% (v/v) EtOAc in PE gave two products in the following order:

(a) $[1\beta,3\alpha\text{-}^2\text{H}_2]\text{-}\beta$ -chloro GA_{20} methyl ester 13-acetate (Fig. 5, [26]) (470 mg) containing 1.81 deuterium atoms per molecule; $^1\text{H-NMR}$ (CDCl_3), δ -values (assignments) 1.18(s, 18- H_3), 2.02(s, OCOCH_3), 2.70(d, $J = 11$ Hz, 6-H), 3.28(d, $J = 11$ Hz, 5-H), 3.73(s, OCH_3), and 4.99 and 5.16(each br, 17- H_2); MS, m/z values (relative intensities) 424(M^+ , 26%), 346(6), 314(2), 303(23), 287(9), 286(8), 43(100), and 29(6).

(b) $[1\beta,3\text{-}^2\text{H}_2]\text{GA}_5$ methyl ester 13-acetate (Fig. 5, [27]) (181 mg) containing 1.69 deuterium atoms per molecule; $^1\text{H-NMR}$ (CDCl_3), δ -values (assignments) 1.22(s, 18- H_3), 2.02(s, OCOCH_3), 2.66(d, $J = 10$ Hz, 6-H), 2.80(d, $J = 10$ Hz, 5-H), 3.72(s, OCH_3), 4.98 and 5.12(each br, 17- H_2), and 5.79(d, $J = 3$ Hz, 2-H); MS, m/z values (relative intensities) 360(M^+ , 28, 63%), 243(15), 242(10), 169(7), 159(8), 158(11), 138(4), 107(15), and 43(100). By heating under reflux for 3.5 h with 0.5 mL 1,8-diazabicyclo[5.4.0]undec-7-ene in 3.5 mL pyridine, 470 mg of the β -chloro-compound was converted into 287 mg of the GA_5 -derivative [27] that was purified by flash chromatography eluting with 18% (v/v) EtOAc in PE. A portion of $[1\beta,3\text{-}^2\text{H}_2]\text{GA}_5$ methyl ester 13-acetate (200 mg) was stirred overnight in 10 mL methanol and 2 mL aqueous K_2CO_3 (saturated solution, diluted $\times 20$). The methanol was evaporated under vacuum. The standard work-up and flash chromatography with 20 to 60% (v/v) EtOAc in PE gave 109 mg $[1\beta,3\text{-}^2\text{H}_2]\text{GA}_5$ methyl ester (Fig. 5, [28]); $^2\text{H-NMR}$ (CHCl_3), δ -values (assignments) 2.31($1\beta\text{-}^2\text{H}$) and 5.70($3\text{-}^2\text{H}$);

Figure 6. A, $^1\text{H-NMR}$ spectrum of GA_5 methyl ester (CDCl_3); B, $^2\text{H-NMR}$ spectrum of $[1\beta,3\text{-}^2\text{H}_2]\text{GA}_5$ methyl ester (CHCl_3); C, $^2\text{H-NMR}$ spectrum of $[1\alpha\text{-}^2\text{H}]\text{GA}_5$ methyl ester (CHCl_3).

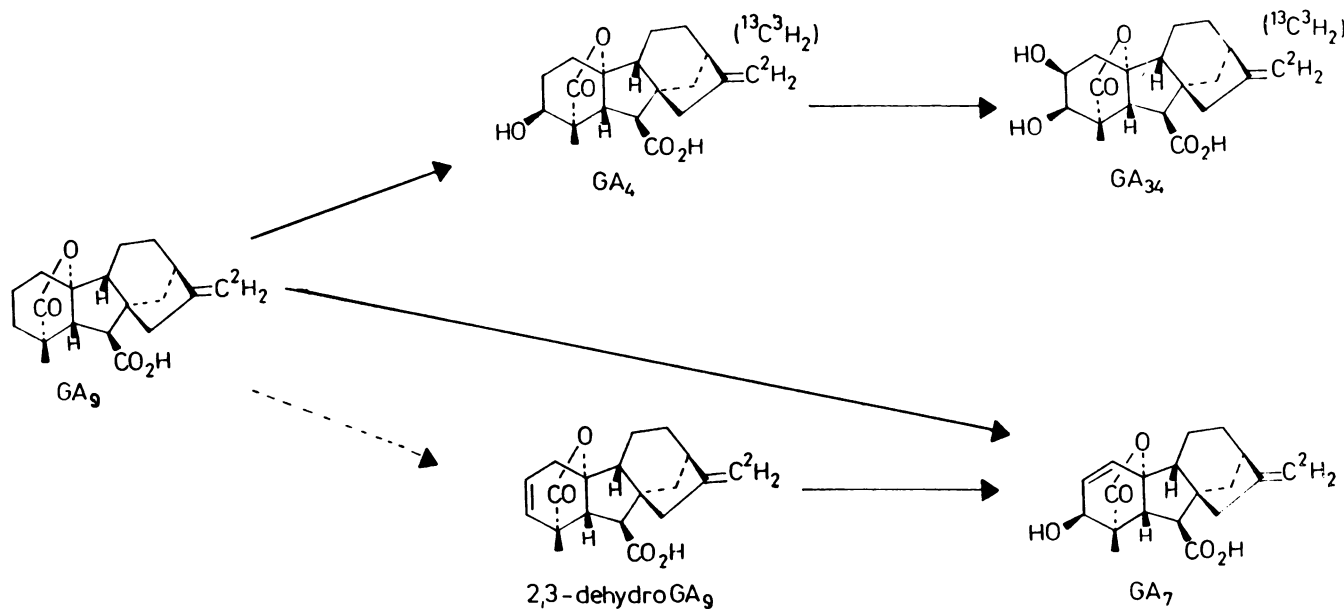


Figure 7. Metabolism of $[17\text{-}^2\text{H}_2]\text{GA}_9$, $[17\text{-}^2\text{H}_2]2,3\text{-dehydroGA}_9$ and $[17\text{-}^{13}\text{C},^3\text{H}_2]\text{GA}_4$ in the cell-free preparations from seeds of *Marah macrocarpus*.

^1H -NMR (CDCl_3), δ -values (assignments) 1.23(s, 18- H_3), 2.65(d, $J = 10$ Hz, 6-H), 2.79(d, $J = 10$ Hz, 5-H), 3.72(s, OCH_3), 4.95 and 5.24(each br, 17- H_2) and 5.80(d, $J = 4$ Hz, 2-H). $[1\beta,3\text{-}^2\text{H}_2]\text{GA}_5$ methyl ester (87 mg) was hydrolyzed by heating under reflux for 16 h with 5 mL 10 M NaOH. The standard work-up and flash chromatography eluting with 50 to 60% (v/v) EtOAc in PE containing 0.1% (v/v) $\text{CH}_3\text{CO}_2\text{H}$ gave 76 mg $[1\beta,3\text{-}^2\text{H}_2]\text{GA}_5$ (Fig. 5, [29]), containing 1.7 deuterium atoms per molecule; ^1H -NMR (CD_3COCD_3), δ -values (assignments) 1.19(s, 18- H_3), 2.55(d, $J = 10$ Hz, 5-H), 2.75(d, $J = 10$ Hz, 6-H), 4.87 and 5.19(each br, 17- H_2) and 5.85(d, $J = 3$ Hz, 2-H); MS, m/z values (relative intensities) 332(M^+ , 25%), 288(100), 227(15), 226(10), 153(59), 143(17), 142(12), 122(14), and 91(44).

$[1\beta,3\text{-}^2\text{H}_2]\text{-}2,3\text{-Dehydrogibberellin A}_9$

$[1\beta,3\text{-}^2\text{H}_2]\text{GA}_5$ methyl ester [28] (109 mg), prepared as described in section 4, was dissolved in 5 mL THF, 0.5 mL methyl oxalyl chloride, and 0.5 mL pyridine, and the solution was heated under reflux for 30 min. The standard work-up gave 120 mg of the crude methyl oxalyl ester (Fig. 5, [30]), a 100 mg portion of which was heated for 1.5 h in 5 mL refluxing toluene containing 1.0 mL tri-*n*-butylstannane and 100 mg 2,2'-azobis(2-methylpropionitrile). Removal of the solvent under vacuum and flash chromatography of the product with 10 to 18% (v/v) EtOAc in PE gave a 164 mg mixture of tin residues and $[1\beta,3\text{-}^2\text{H}_2]\text{-}2,3\text{-dehydroGA}_9$ methyl ester (Fig. 5, [31]) containing 1.70 deuterium atoms per molecule;

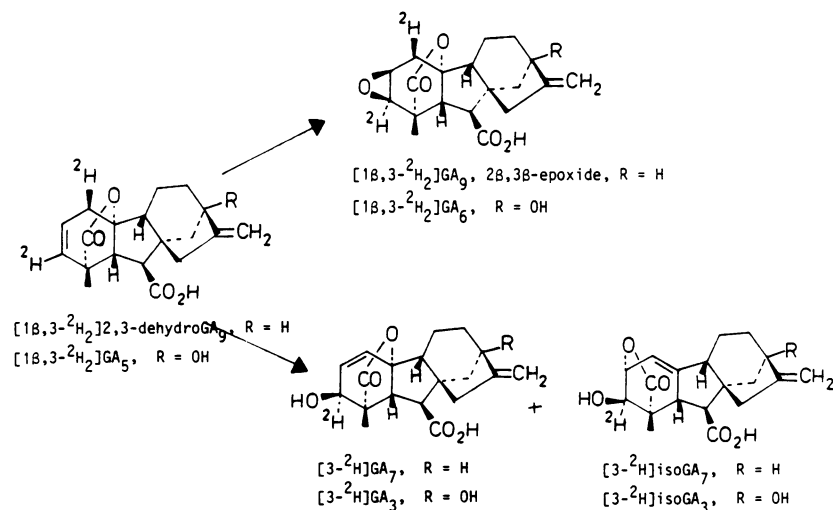


Figure 8. Metabolism of $[1\beta,3\text{-}^2\text{H}_2]2,3\text{-dehydroGA}_9$ and $[1\beta,3\text{-}^2\text{H}_2]\text{GA}_5$ in the cell-free preparation from seeds of *Marah macrocarpus*.

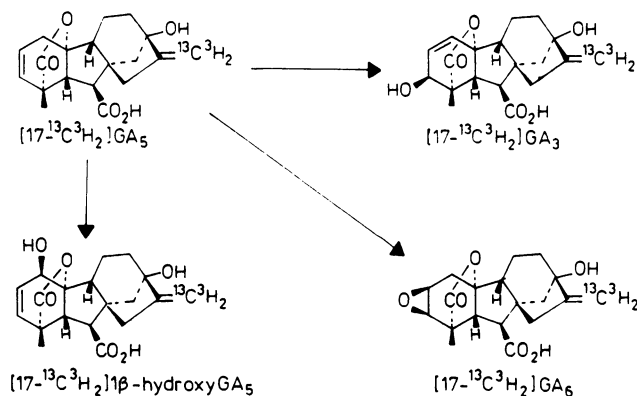


Figure 9. Metabolism of $[17-^{13}\text{C}_3\text{H}_2]\text{GA}_5$ by the cell-free preparation from seeds of *Marah macrocarpus*.

$^1\text{H-NMR}$ (CDCl_3), δ -values (assignments) 1.18(s, 18- H_3), 2.66(d, $J = 10$ Hz, 6-H), 2.81(d, $J = 10$ Hz, 5-H), 3.72(s, OCH_3), 4.86 and 4.98(each br, 17- H_2) and 5.80(d, $J = 3$ Hz, 2-H). This crude methyl ester (60 mg) in 5 mL 10 M NaOH was heated under reflux for 16 h. After the standard work-up, the recovered product in methanol was treated (pH 1–2) with 3 M hydrochloric acid (to relactonize). The standard work-up and flash chromatography eluting with 30 to 40% (v/v) EtOAc in PE containing 0.1% (v/v) $\text{CH}_3\text{CO}_2\text{H}$ gave 36 mg of pure $[1\beta,3-^2\text{H}_2]\text{dehydroGA}_9$ (Fig. 5, [32]), containing 1.7 deuterium atoms per molecule; $^1\text{H-NMR}$ (CD_3COCD_3), δ -values (assignments) 1.19(s, 18- H_3), 2.68(d, $J = 10.5$ Hz, 6-H), 2.75(d, $J = 10.5$ Hz, 5-H), 4.86 and 4.98 (each br, 17- H_2) and 5.80(d, $J = 4$ Hz, 2-H); MS, m/z values (relative intensities) 272($\text{M}^+ - 44$, 100%), 243(9), 242(5), 169(9), 159(11), 158(14), 138(10), and 107(24).

RESULTS

Synthesis of Substrates

Of the eight labeled substrates used, $[17-^2\text{H}_2]\text{GA}_9$, $[17-^2\text{H}_2]\text{-2,3-dehydroGA}_9$, $[1\alpha-^2\text{H}_1]\text{GA}_5$, $[1\beta,3-^2\text{H}_2]\text{-2,3-dehydroGA}_9$, and $[1\beta,3-^2\text{H}_2]\text{GA}_5$ have not been synthesized previously. $[17-^2\text{H}_2]\text{GA}_9$ (Fig. 2) and $[17-^2\text{H}_2]\text{-2,3-dehydroGA}_9$ (Fig. 3) were prepared from their corresponding norketones using the Lombardo method (21).

The method of synthesis of $[1\alpha-^2\text{H}_1]\text{GA}_5$ (Fig. 4, [23]) from GA_3 is adapted from Murofushi et al. (26). Controlled catalytic reduction of GA_3 methyl ester 13-acetate 3-methanesulfonate (Fig. 4, [18]) with deuterium gas and a partially poisoned catalyst (24), gave an inseparable mixture of $[1\alpha-^2\text{H}_1]\text{-GA}_5$ methyl ester 13-acetate (Fig. 4, [19]) and its 1,2-double-bond isomer (Fig. 4, [20]). Following the procedure of Bearder et al. (7) the mixture of isomers [19] and [20] was treated with toluene-4-sulfonic acid and acetic anhydride to convert [20] to the 19,2-lactone (Fig. 4, [21]) from which the unchanged $[1\alpha-^2\text{H}_1]\text{GA}_5$ methyl ester 13-acetate was separated by flash chromatography. Stepwise hydrolysis of the 13-acetate, then the methyl ester (Fig. 4, [22]), gave the required $[1\alpha-^2\text{H}_1]\text{GA}_5$ containing 0.5 atoms of deuterium per molecule.

The synthesis of $[1\beta,3-^2\text{H}_2]\text{GA}_5$ (Fig. 5, [29]) is adapted from Beale et al. (4). Reduction of the enone (Fig. 5, [24]) as described by Beale and MacMillan (3) gave a mixture of the two epimers (Fig. 5, [25]), which were treated with phosphoryl chloride in pyridine to give the chloro-compound (Fig. 5, [26]) and the 2,3-ene (Fig. 5, [27]). Treatment of the chloro-compound with 1,8-diazabicyclo[5.4.0]undec-7-ene and pyridine gave the 2,3-ene, which was stepwise hydrolyzed to the methyl ester (Fig. 5, [28]) then to $[1\beta,3-^2\text{H}_2]\text{GA}_5$ (Fig. 5, [29]) containing 1.69 atoms of deuterium per molecule. The corresponding $[1\beta,3-^2\text{H}_2]\text{-2,3-dehydroGA}_9$ (Fig. 5, [32]) was prepared by 13-deoxygenation of $[1\beta,3-^2\text{H}_2]\text{GA}_5$ methyl ester via the methyloxalyl ester (Fig. 5, [30]) by the method of Dolan and MacMillan (11). Hydrolysis of the resultant methyl ester (Fig. 5, [31]) gave $[1\beta,3-^2\text{H}_2]\text{-2,3-dehydroGA}_9$ containing 1.70 atoms of deuterium per molecule.

The stereochemistry of the deuterium labels in these substrates was determined by $^2\text{H-NMR}$ (1). We (23) had previously established by NMR that the borodeuteride reduction of enones, e.g. [24] (Fig. 5) introduces a 1β -deuterium in the reduction products, e.g. [25] (Fig. 5). The stereochemistry of $[1\beta,3-^2\text{H}_2]\text{GA}_5$ (Fig. 5, [29]) and $[1\beta,3-^2\text{H}_2]\text{-2,3-dehydroGA}_9$ (Fig. 5, [32]) therefore follows by analogy, and the $^2\text{H-NMR}$ spectrum (Fig. 6B) of $[1\beta,3-^2\text{H}_2]\text{GA}_5$ methyl ester (Fig. 5, [28]) showed two signals at $\delta 2.31$ and $\delta 5.70$, which can therefore be attributed to the 1β - and 3-deuterons, respectively. The assignments shown in Figure 6A can therefore be made for the 1β -, 1α -, 2-, and 3-hydrogen signals in the $^1\text{H-NMR}$ spectrum of unlabeled GA_5 methyl ester; they were confirmed by decoupling experiments. The $^2\text{H-NMR}$ spectrum (Fig. 6C) of the prepared $[1\alpha-^2\text{H}_1]\text{GA}_5$ methyl ester (Fig. 4, [22]) showed one signal at $\delta 2.56$ with a possible slight shoulder at $\delta 2.31$ from which it may be conservatively concluded that the substrate $[1\alpha-^2\text{H}_1]\text{GA}_5$ (Fig. 4, [23]) is at least 80% stereospecifically labeled with a 1α -deuterium.

Marah System

The metabolites from the eight substrates were identified (Table I) by full scan GC-MS and KRI data except for $[17-$

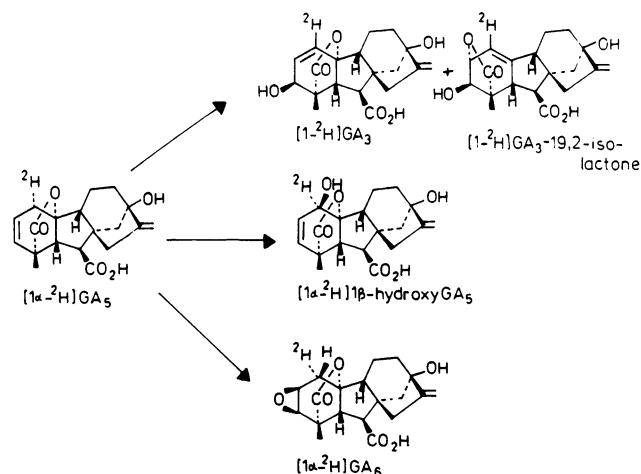


Figure 10. Metabolism of $[1\alpha-^2\text{H}_1]\text{GA}_5$ by the cell-free preparation from seeds of *Marah macrocarpus*.

Table 1. GC-MS Data
Identification of metabolites from incubations of labeled substrates with the *Marah* cell-free preparation.

Substrate	Metabolite (KRI)	m/z (relative intensities by GC-MS)										
[1 β ,3- ² H ₂]2,3-dehydro GA ₉	[3 α - ² H] GA ₇ (2525)	417 (M ⁺ , 1%)	385 (5)	357 (10)	288 (7)	282 (16)	223 (100)	207 (40)	156 (22)	75 (100)	73 (41)	
	[3 α - ² H] iso GA ₇ (2483)	357 (5%)	299 (5)	281 (8)	223 (43)	208 (7)	180 (5)	156 (11)	130 (4)	113 (4)	75 (100)	
	[1 β ,3 α - ² H] 2 β ,3 β -epoxy GA ₉ (2402)	314 (M ⁺ , 80%)	281 (8)	241 (95)	224 (100)	198 (27)	181 (35)	157 (47)	130 (13)	113 (26)	91 (17)	
[17- ² H ₂]2,3-dehydro GA ₉	[17- ² H ₂] GA ₇ (2525)	418 (M ⁺ , 1%)	386 (1)	358 (7)	313 (5)	300 (7)	225 (37)	224 (67)	155 (19)	75 (100)	73 (42)	
	[17- ² H ₂] GA ₄ (2503)	420 (M ⁺ , 5%)	388 (13)	360 (2)	330 (17)	291 (31)	286 (100)	227 (59)	129 (31)	75 (28)	73 (56)	
	[1 α - ² H] GA ₅	505 (M ⁺ , 53%)	371 (40)	355 (20)	311 (20)	281 (13)	238 (36)	221 (16)	167 (30)	95 (90)	73 (100)	
[1 α - ² H] GA ₅	[1- ² H] iso GA ₃ (2628)	505 (M ⁺ , 5%)	371 (6)	355 (3)	281 (5)	238 (1)	221 (1)	289 (6)	75 (100)	73 (66)		
	[1 α - ² H] 1 β -hydroxy GA ₅ (2588)	361 (16%)	341 (13)	282 (10)	267 (10)	217 (20)	207 (73)	183 (13)	149 (10)	109 (30)	73 (100)	
	[1 α - ² H] GA ₆ (2574)	433 (M ⁺ , 3%)	281 (13)	259 (3)	207 (26)	171 (13)	115 (13)	93 (10)	75 (100)	73 (43)		
[17- ¹³ C ³ H ₂] GA ₅	[17- ¹³ C] GA ₃ (2669)	505 (M ⁺ , 100%)	371 (37)	356 (17)	297 (14)	282 (11)	239 (31)	222 (13)	209 (13)	194 (13)	75 (49)	
	[17- ¹³ C] 1 β -hydroxy GA ₅ (2588)	505 (M ⁺ , 60%)	388 (56)	371 (10)	342 (16)	312 (16)	281 (23)	221 (30)	207 (50)	93 (23)	76 (13)	
	[17- ¹³ C] GA ₅ (2574)	433 (M ⁺ , 43%)	304 (46)	282 (3)	236 (10)	221 (10)	208 (36)	171 (100)	135 (10)	93 (23)	76 (13)	
[17- ¹³ C ³ H ₂] GA ₂₀	[17- ¹³ C] GA ₁ (2669)	507 (M ⁺ , 100%)	492 (10)	449 (30)	377 (13)	314 (10)	236 (10)	208 (36)	181 (13)	73 (86)		
	[17- ¹³ C] GA ₂₀ (2575)	507 (M ⁺ , 100%)	448 (4)	376 (61)	287 (2)	231 (2)	195 (56)	180 (5)	153 (5)			
	[17- ¹³ C] GA ₂₀ (2684)	by SIM and Rt ¹³ C ₆ : ¹³ C ₁ = 19.9:80.1										
[17- ¹³ C ³ H ₂] GA ₄	[17- ¹³ C] GA ₅ (2818)	by SIM and Rt ¹³ C ₆ : ¹³ C ₁ = 14.7:85.3										
	[17- ¹³ C] GA ₃ (2665)	507 (M ⁺ , 43%)	431 (20)	356 (10)	269 (20)	217 (36)	208 (13)	147 (33)	129 (20)	75 (100)	73 (80)	
	[1 β ,3- ³ H ₂] GA ₅	[1 β ,3 α - ² H ₂] GA ₆ (2574)	434 (M ⁺ , 73%)	419 (6)	371 (16)	304 (16)	281 (6)	235 (33)	208 (23)	194 (13)	181 (6)	73 (100)
[1 β ,3- ³ H ₂] GA ₅	[3 α - ² H] GA ₃ (2692)	505 (M ⁺ , 40%)	371 (40)	356 (17)	312 (20)	238 (16)	222 (10)	207 (15)	193 (10)	167 (6)	75 (100)	
	[3 α - ² H] iso GA ₃ (2628)	505 (M ⁺ , 26%)	371 (40)	356 (13)	238 (10)	221 (10)	194 (10)	167 (13)	75 (100)	73 (59)		
	[17- ² H ₂] GA ₄ (2503)	420 (M ⁺ , 11%)	388 (13)	330 (22)	286 (79)	263 (22)	226 (100)	203 (32)	169 (25)	131 (20)	75 (60)	
[17- ² H ₂] GA ₉	[17- ² H ₂] GA ₇ (2525)	418 (M ⁺ , 5%)	386 (8)	300 (17)	244 (25)	224 (92)	195 (28)	181 (30)	157 (16)	131 (11)	75 (100)	
	[17- ² H ₂] GA ₃ (2665)	508 (M ⁺ , 21%)	474 (3)	357 (4)	311 (10)	283 (7)	269 (9)	209 (13)	169 (13)	75 (87)	73 (100)	

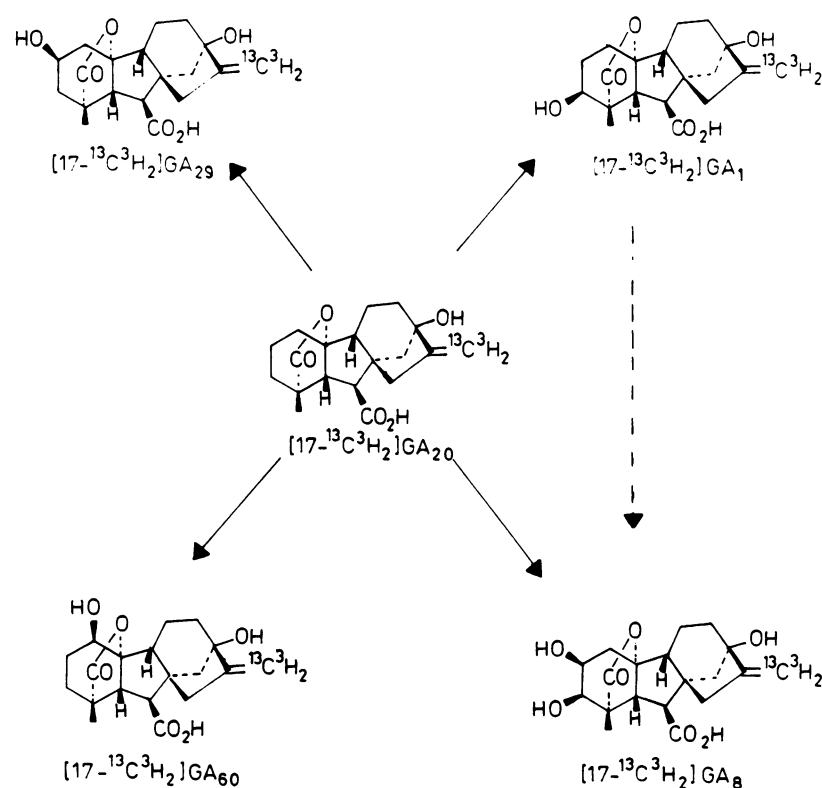


Figure 11. Metabolism of $[17\text{-}^{13}\text{C},^3\text{H}_2]\text{GA}_{20}$ by the cell-free preparation from seeds of *Marah macrocarpus*. The broken arrow denotes a step not directly established in the present study.

$^{13}\text{C}]\text{GA}_{29}$ and $[17\text{-}^{13}\text{C}]\text{GA}_8$ from $[17\text{-}^{13}\text{C},^3\text{H}_2]\text{GA}_{20}$, where the identification was based on GC-SIM and Rt data.

The results from the feeds of $[17\text{-}^2\text{H}_2]\text{GA}_9$ (Fig. 2, [13]), $[17\text{-}^2\text{H}_2]\text{-2,3-dehydroGA}_9$ (Fig. 3, [17]), and $[17\text{-}^{13}\text{C},^3\text{H}_2]\text{GA}_4$ are shown in Figure 7. $[17\text{-}^2\text{H}_2]\text{GA}_7$ was formed from $[17\text{-}^2\text{H}_2]\text{-GA}_9$ and $[17\text{-}^2\text{H}_2]\text{-2,3-dehydroGA}_9$, but $[17\text{-}^{13}\text{C},^3\text{H}_2]\text{GA}_7$ was not formed from $[17\text{-}^{13}\text{C},^3\text{H}_2]\text{GA}_4$. The metabolism of $[17\text{-}^2\text{H}_2]\text{-2,3-dehydroGA}_9$ to $[17\text{-}^2\text{H}_2]\text{GA}_7$ appeared to be quantitative based on the facts that no substrate remained and no other metabolites were detected. The conversion of $[17\text{-}^2\text{H}_2]\text{-GA}_9$ to $[17\text{-}^2\text{H}_2]\text{GA}_7$ was low and $[17\text{-}^2\text{H}_2]\text{GA}_4$ and $[17\text{-}^2\text{H}_2]\text{-GA}_{34}$ were also formed. $[17\text{-}^{13}\text{C},^3\text{H}_2]\text{GA}_4$ gave only $[^{13}\text{C}]\text{GA}_{34}$.

These results establish two separate branch pathways from GA_9 , one to GA_4 and GA_{34} , and the other to GA_7 , probably via 2,3-dehydro GA_9 . The conversion of 2,3-dehydro GA_9 to GA_7 involves the loss of one of the hydrogens at carbon-1. To determine the stereochemistry of the loss, $[1\beta,3\text{-}^2\text{H}_2]\text{-2,3-dehydroGA}_9$ (Fig. 5, [32]) was synthesized and incubated in the *Marah* system. As shown in Figure 8, the major products were $[3\alpha\text{-}^2\text{H}_1]\text{GA}_7$ and $[3\alpha\text{-}^2\text{H}_1]\text{GA}_7$ 19,2-isomeric lactone (a GC artifact from GA_7); a minor product was $[1\beta,3\alpha\text{-}^2\text{H}_2]\text{GA}_9$ 2,3-epoxide. This result shows that the 1β -hydrogen is lost from 2,3-dehydro GA_9 in the conversion to GA_7 .

Interestingly, the *Marah* system also catalyzed the metab-

Table II. GC-MS Data

Identification of metabolites from incubation of $[1\beta,2\beta\text{-}^2\text{H}_2]\text{GA}_4$ in cultures of *Gibberella fujikuroi*, mutant B1-41a.

Metabolite	KRI	^2H -Atoms per Molecule ^a		m/z (Relative Intensities by GC-MS)
		+AM01618	-AM01618	
$[1,2\text{-}^2\text{H}_2]\text{GA}_7$	2525	ND	ND	418(M^+ , 3%), 386(13), 358(12), 313(6), 264(5), 224(5), 206(7), 157(22), and 44(44)
$[1,2\text{-}^2\text{H}_2]\text{GA}_3$	2692	1.63	1.45	506(M^+ , 48%), 491(4), 391(6), 372(19), 357(10), 313(6), 238(12), 207(7), 167(6), 143(3), and 44(32)
$[1,2\text{-}^2\text{H}_2]\text{GA}_1$	2669	1.62	1.50	508(M^+ , 39%), 493(3), 385(13), 357(10), 298(9), 289(42), 239(14), 207(22), 143(97), and 44(83)

^a The substrate, $[1\beta,2\beta\text{-}^2\text{H}_2]\text{GA}_4$, contained 1.65 atoms ^2H per molecule.

olism of GA₅, the 13-hydroxyl analog of 2,3-dehydroGA₉, to GA₃, the 13-hydroxyl analog of GA₇. Thus, [17-¹³C,³H₂]GA₅ was metabolized in high yield to [17-¹³C]GA₃ as shown in Figure 9; the only other products were trace amounts of [17-¹³C]GA₆ and [17-¹³C]-1β-hydroxyGA₅. When unlabeled GA₆ was incubated in the system, no GA₃ was formed and only unmetabolized GA₆ was detected. Thus, GA₆ does not appear to be an intermediate between GA₅ and GA₃. Like the metabolism of 2,3-dehydroGA₉ to GA₇, the metabolism of GA₅ to GA₃ occurs with the loss of the 1β-hydrogen from GA₅ as shown by the following evidence. First, [1β,3-²H₂]GA₅ (Fig. 5, [29]) was metabolized mainly to [3α-²H₁]GA₃ and its GC-artifact, [3α-²H₁]GA₃ 19,2-isomeric lactone, together with a minor amount of [1β,3α-²H₂]GA₆ (Fig. 8). Second, [1α-²H₁]GA₅ (Fig. 4, [23]) was metabolized to [1α-²H₁]GA₃ and the isomeric lactone (Fig. 10), *i.e.* the 1α-²H₁ was retained; traces of [1α-²H₁]-1β-hydroxyGA₅ and [1α-²H₁]GA₆ were also formed.

In contrast to the metabolism of GA₉ to GA₇, GA₂₀ was not metabolized to GA₃ in the *Marah* system. As shown in Figure 11, incubation of [17-¹³C,³H₂]GA₂₀ gave [17-¹³C]-labeled GA₁, GA₈, GA₂₉, and GA₆₀, but no [17-¹³C]GA₃.

Malus System

As with the *Marah* system (see Fig. 7), [17-²H₂]-2,3-dehydroGA₉ was metabolized to [17-²H₂]GA₇, and [17-²H₂]GA₉ was converted into both [17-²H₂]GA₇ and [17-²H₂]GA₄. No metabolites were detected from [17-¹³C,³H₂]GA₂₀. [17-¹³C,³H₂]GA₅ was metabolized to [17-¹³C]GA₃, but only in 0.1% yield, and to [17-¹³C]-1β-hydroxyGA₅ in trace amounts (0.02%).

Fungal System

In cultures of *G. fujikuroi*, mutant B1-41a, containing AMO 1618, [1β,2β-²H₂]GA₄ was converted into [2H₂]GA₇, [2H₂]GA₃, and [2H₂]GA₁ (Table II) showing that 1,2 dehydrogenation of GA₄ to GA₇, and hence GA₃, occurs with loss of the 1α- and 2α-hydrogens.

DISCUSSION

The results of this study indicate that GA₇ is formed from GA₉ via 2,3-dehydroGA₉ in seeds of *Marah macrocarpus* and *Malus domestica* and that GA₄ is not a precursor of GA₇ in these systems. The absence of 2,3-dehydroGA₉ as a metabolite of GA₉ is probably due to the observed efficient conversion of 2,3-dehydroGA₉ to GA₇ by these cell-free preparations.

In *Marah* and *Malus*, the branch pathway GA₉ → 2,3-dehydroGA₉ → GA₇ parallels the corresponding 13-hydroxy branch pathway, GA₂₀ → GA₅ → GA₃ recently established (15) in maize shoots. Moreover, the cell-free system from *Marah* seeds catalyzed the conversion of GA₅ to GA₃ in high yield.

The stereochemistry of the conversion of 2,3-dehydroGA₉ to GA₇ and of GA₅ to GA₃ was shown to involve the loss of the 1β-hydrogen. We (2) have previously shown that GA₂₀ is

converted into GA₅ with the loss of the 2β- and 3β-hydrogens by an enzyme preparation from seeds of *Phaseolus vulgaris*. Thus, our hypothesis that the 1,2-double bond in GA₇ and GA₃ originates in higher plants by the loss of the 1β- and 2β-hydrogens appears to be correct. Furthermore, the biosynthesis of GA₃ in maize shoots (15) and in *Marah* seeds (this paper) and of GA₇ in *Marah* and *Malus* seeds (this paper) differs from the biosynthesis of GA₃ and GA₇ in the fungus in the following respects: in these higher plant systems, GA₃ and GA₇ are formed by separate pathways; in the fungus, GA₃ is formed from GA₇ in a single linear pathway. These results imply that higher plants have not acquired the genes for GA biosynthesis from the fungus.

The *Marah* enzyme preparation is 2-oxoglutarate dependent (28) and the observed conversions of GA₅ to GA₃ and of 2,3-dehydroGA₉ to GA₇ are novel properties of such oxygenases. They are reminiscent of allylic oxidation by singlet dioxygen as suggested by MacMillan *et al.* (25). The cell-free preparations from *Marah* and *Malus* seeds also contain 2,3-epoxidase activity which is more apparent when the 2,3-dehydroGA₉ and GA₅ are deuterated at position-1. However, the epoxides do not appear to be intermediates between the 2,3-enes and the 1-en-3-ols since GA₆ was not metabolized to GA₃ in the *Marah* system. Studies on the mechanism of the formation of GA₃ and GA₇ and 2,3-dehydroGA₉ must await purification of the enzyme(s).

ACKNOWLEDGMENTS

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