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

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**ABSTRACT**

Cell-free preparations from seeds of *Marah macrocarpus* L. and *Malus domestica* L. catalyzed the conversion of gibberellin A₃ (GA₃) and 2,3-dehydroGA₉ to GA₆; GA₃ was also metabolized to GA₆ in a branch pathway. The preparation from *Marah* seeds also metabolized GA₃ to GA₆ in high yield; GA₃ was a minor product and was not metabolized to GA₆. Using substrates stereospecifically labeled with deuterium, it was shown that the metabolism of GA₃ to GA₆ and of 2,3-dehydroGA₉ to GA₆ occurs with the loss of the 1β-hydrogen. In cultures of *Gibberella fujikuroi*, mutant B1-41a, [1β,2β-²H₂]GA₉ was metabolized to [1,2-²H₂]GA₆ with the loss of the 1α- and 2α-hydrogens. These results provide further evidence that the biosynthetic origin of GA₃ and GA₆ 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 GA₉ (Fig. 1, [5]). The stereochemistry of the 1,2-didehydrogenation of GA₉ (Fig. 1, [1]) to GA₃ in this fungus was first investigated by Evans et al. (12). These workers concluded from incorporations of tritium from 2R-, 2S-, and 5R-²H₂-labeled [2-¹⁴C]-mevalonics that the 1α- and 2α-hydrogens of GA₉ 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-²H₂]-GA₄ (Fig. 1, [2]) (22) was metabolized to GA₇ with retention of the deuterium label. Using the same mutant, the present paper reports the metabolism of [1β,2β-²H₂]GA₉ (Fig. 1, [3]) (23) into GA₉ with retention of both deuterium labels. Thus, it has been firmly established that GA₇ can be converted into GA₉ 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 GA₉ 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 GA₇ in higher plants had not been conclusively established. However, GA₇ has been identified by GC-MS in extracts 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 GA₆ and the stereochemistry of the process. Attempts over many seasons failed to provide evidence for the enzymatic formation of GA₇ by cell-free preparations that metabolized GA₃ to GA₇. The recent findings of Fujioka et al. (14, 15) established the natural occurrence of GA₇ in shoots of *Zea mays* L. and the biosynthesis of GA₇ from GA₉ (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 GA₉ but from GA₃ 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 GA₇ from GA₉ 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-SIM 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.
methyl-N-trimethylsilyltrifluoroacetamide (Pierce and Warner, 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.


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 [1β,2β-2H2]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 testa. 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 x 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) [17-13C,1H2]-GA5; prepared by Fujioka et al. (13); (b) [17-13C,1H2]GA30; prepared by Ingram et al. (20); (c) [17-13C,1H2]GA4; prepared by Dr. M. H. Beale (unpublished); (d) [17-2H2]GA4; (e) [17-2H2]-2,3-dehydroGA4; (f) [1α-2H]GA5; (g) [1β,3-2H2]GA5; and (h) [1β,3-2H2]-2,3-dehydroGA5. The preparation of substrates (d) to (h) is described in the following paragraphs.


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.](image-url)
gas at room temperature for 1 h with 5 mL of methylenating reagent, prepared (21) from 1.14 g zinc powder, C₂H₂Br₂ (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 THF gave 23 mg [17-²H₂]GA₉ methyl ester (Fig. 2, [12]; 1H-NMR (CDCl₃), 6-values (assignments) 1.08(s, 18-H₃), 2.54(d, J = 11 Hz, 5-H), 2.71(d, J = 10 Hz, 6-H), and 3.70(s, OCH₃); 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 [17-²H₂]-GA₉ (Fig. 2, [13]) containing 1.70 deuterium atoms per molecule; 1H-NMR (CDCl₃), 6-values (assignments) 1.13(s, 18-H₃), 2.50(d, J = 10 Hz, 5-H), and 2.74(d, J = 10 Hz, 6-H).

[17-²H₂]₂,3-Dehydrogibberellin A₈

A mixture (6 g) of GA₄ (Fig. 1) (70%) and GA₇ (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) GA₄ 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 phosphorus 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-dehydroGA₈ methyl ester norketone (Fig. 3, [15]); 1H-NMR (CDCl₃), 6-values (assignments) 1.24(s, 18-H₃), 2.69(d, J = 10 Hz, 5-H), 2.82(d, J = 10 Hz, 6-H), 3.74(s, OCH₃), 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 C₂H₂Br₂ (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 [17-²H₂]-2,3-dehydroGA₈ methyl ester (Fig. 3, [16]) containing 1.70 deuterium atoms per molecule; 1H-NMR (CDCl₃), 6-values (assignments) 1.22(s, 18-H₃), 2.66(d, J = 10 Hz, 5-H), 2.79(d, J = 10 Hz, 6-H), 3.71(s, CO₂CH₃), 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 relactonize). 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 [17-²H₂]-2,3-dehydroGA₈ (Fig. 3, [17]) containing 1.67 deuterium atoms per molecule; 1H-NMR (CDCl₃), 6-values (assignments) 1.24(s, 18-H₃), 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).

[1α-²H₆]-Gibberellin A₅

The known (22) GA₅ 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 [2-²H₂]-labeled GA₅ 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. ¹H(NMR)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 [1α-²H₁]GA₅, methyl ester 13-acetate (Fig. 4, [19]). Fractions 14 to 16 contained 47 mg of a 1:1 mixture of [1α-²H₁]GA₅ 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 2. Synthesis of [17-²H₂]GA₉. Reagents: (i) Zn, C₂H₂Br₂, TiCl₄, THF; (ii) 10 M NaOH.

Figure 3. Synthesis of [17-²H₂]₂,3-dehydroGA₈. Reagents: (i) POCl₃, pyridine; (ii) Zn, C₂H₂Br₂, TiCl₄, THF; (iii) 10 M NaOH.
BIOSYNTHETIC ORIGIN OF GA₃ AND GA₇

Figure 4. Preparation of [1α-²H₁]GA₅. Reagents: (i) D₂, 10% Pd on CaCO₃, cyclohexene, C₃D₈N, EtOAc; (ii) p-MeC₆H₄SO₃H, (MeCO)O; (iii) K₂CO₃, 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 [1α-²H₁]GA₅ methyl ester 13-acetate containing 0.55 deuterium atoms per molecule: 1H-NMR (CDCl₃), δ-value (assignment) 2.56(1α-²H₁); 1H-NMR (CDCl₃), δ-values (assignments) 1.22(s, 18-H₃), 2.02(s, 13-OCOCH₃), 2.66(d, J = 10 Hz, 5-H), 2.80(d, J = 10 Hz, 6-H), 3.72(s, OCH₃), and 5.80(dd, J = 2.5 and 10.5 Hz, 2-H); MS, m/z values (relative intensities) 387(M⁺, 21%), 356(25), 345(10), 301(10), 283(10), 268(9), 240(9), 222(17), 208(17), and 194(7). Next, 20 mg of [1α-²H₁]GA₅ methyl ester 13-acetate in 5 mL methanol was stirred for 16 h with 5 mL aqueous potassium carbonate (saturated solution diluted × 20). The standard work-up, followed by crystallization from EtOAc-PE, gave 13.5 mg [1α-²H₁]GA₅ methyl ester (Fig. 4, [22]), containing 0.53 deuterium atoms per molecule; 1H-NMR (CDCl₃), δ-value (assignment) 2.57(1α-²H₁); 1H-NMR (CDCl₃), δ-values (assignments) 1.23(s, 18-H₃), 2.66(d, J = 10 Hz, 5-H), 2.79(d, J = 10 Hz, 6-H), 3.73(s, OCH₃), 4.96 and 5.25(each br, 17-HH₂), 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 [1α-²H₁]GA₅ 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 [1β,3-²H₂]GA₅ and [1β,3-²H₂]-2,3-dehydroGA₅. Reagents: (i) NaB₄H₄, Cu(II)Cl, MeOH; (ii) POCl₃, pyridine; (iii) DBU, pyridine; (iv) K₂CO₃, MeOH-H₂O (5:1); (v) 10 M NaOH; (vi) MeO₂C-COCI, THF, pyridine; (vii) nBu₃SnH, AIBN, C₆H₅Me.
chromatography and elution with 50 to 60% (v/v) EtOAc in PE containing 0.1% (v/v) CH₂CO₂H, gave 2.8 mg [1α-3H]-GA₃ (Fig. 4, [23]) containing 0.50 deuterium atoms per molecule; ¹H-NMR (CD₂COCD₃), δ-values (assignments) 1.19(s, 18-H₃), 2.55(d, J = 10 Hz, 5-H), 2.75(d, J = 10 Hz, 6-H), 4.86 and 5.19(each br, 17-H₂), 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).

[1β,3-²H₂] Gibberellic A₅

Copper (I) chloride (750 mg) was added to 1.8 g of the known (4) 3-oxoGA₃ methyl ester 13-acetate (Fig. 5, [24]) in 100 mL methanol with stirring at room temperature. After 5 min, 320 mg NaBH₄ 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 3α:3β-alcohols (determined by ¹H-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) [1β,3α-²H₂]-3β-chloroGA₃₀ methyl ester 13-acetate (Fig. 5, [26]) (470 mg) containing 1.81 deuterium atoms per molecule; ¹H-NMR (CDCl₃), δ-values (assignments) 1.18(s, 18-H₃), 2.02(s, OCOCH₃), 2.70(d, J = 11 Hz, 6-H), 3.28(d, J = 11 Hz, 5-H), 3.73(s, OCH₃), and 4.99 and 5.16(each br, 17-H₂); 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) [1β,3-²H₂]GA₅ methyl ester 13-acetate (Fig. 5, [27]) (181 mg) containing 1.69 deuterium atoms per molecule; ¹H-NMR (CDCl₃), δ-values (assignments) 1.22(s, 18-H₃), 2.02(s, OCOCH₃), 2.66(d, J = 10 Hz, 6-H), 2.80(d, J = 10 Hz, 5-H), 3.72(s, OCH₃), 4.98 and 5.12(each br, 17-H₂), 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 3β-chloro-derivative was converted into 287 mg of the GA₅-derivative [27] that was purified by flash chromatography eluting with 18% (v/v) EtOAc in PE. A portion of [1β,3-²H₂]GA₅ methyl ester 13-acetate (200 mg) was stirred overnight in 10 mL methanol and 2 mL aqueous K₂CO₃ (saturated solution, diluted × 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 [1α,3-²H₂]GA₅ methyl ester (Fig. 5, [28]); ¹H-NMR (CHCl₃), δ-values (assignments) 2.31(1β-²H) and 5.70(3-²H);

Figure 6. A, ¹H-NMR spectrum of GA₅ methyl ester (CDCl₃); B, ¹H-NMR spectrum of [1β,3-²H₂]GA₅ methyl ester (CDCl₃); C, ²H-NMR spectrum of [1α-²H]GA₅ methyl ester (CHCl₃).
BIOSYNTHETIC ORIGIN OF GA$_3$ AND GA$_7$

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

$^1$H-NMR (CDCl$_3$), $\delta$-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-$^{13}$H$_2$]GA$_3$ 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) CH$_3$CO$_2$H gave 76 mg [1$\beta$,3-$^{13}$H$_2$]GA$_3$ (Fig. 5, [29]), containing 1.7 deuterium atoms per molecule; $^1$H-NMR (CD$_3$COCD$_3$), $\delta$-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-$^{13}$H$_2$]-2,3-Dehydrogibberellin A$_9$

[1$\beta$,3-$^{13}$H$_2$]GA$_3$ 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-$^{13}$H$_2$]-2,3-dehydroGA$_9$ methyl ester (Fig. 5, [31]) containing 1.70 deuterium atoms per molecule;

Figure 8. Metabolism of [1$\beta$,3-$^{13}$H$_2$]-2,3-dehydroGA$_9$ and [1$\beta$,3-$^{13}$H$_2$]GA$_6$ in the cell-free preparation from seeds of Marah macrocarpus.
1H-NMR (CDCl3), δ-values (assignments) 1.18(s, 18-H3), 2.66(d, J = 10 Hz, 6-H), 3.72(s, OCH3), 4.86 and 4.98(each br, 17-H2) 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) CH3CO2H gave 36 mg of pure [18,3-2H2]dehydroGA9 (Fig. 5, 32), containing 1.7 deuterium atoms per molecule; 1H-NMR (CD2COCD2), δ-values (assignments) 1.19(s, 18-H3), 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-H2) and 5.80(d, J = 4 Hz, 2-H); MS, m/z values (relative intensities) 272(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, [17-2H]GA9, [17-2H2]2,3-dehydroGA9, [1α-2H]GA5, [1β,3-2H2]2,3-dehydroGA9, and [1β,3-2H2]GA9 have not been synthesized previously. [17-2H]GA9 (Fig. 2) and [17-2H2]2,3-dehydroGA9 (Fig. 3) were prepared from their corresponding norketones using the Lombardo method (21).

The method of synthesis of [1α-2H]GA5 (Fig. 4, 23) from GA5 is adapted from Murofushi et al. (26). Controlled catalytic reduction of GA5 methyl ester 13-acetate 3-methanesulfonate (Fig. 4, 18) with deuterium gas and a partially poisoned catalyst (24), gave an inseparable mixture of [1α-2H]GA5 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 [1α-2H]GA5 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 [1α-2H]GA5 containing 0.5 atoms of deuterium per molecule.

The synthesis of [1β,3-2H2]GA9 (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 [1β,3-2H2]GA9 (Fig. 5, 29) containing 1.69 atoms of deuterium per molecule. The corresponding [1β,3-2H2]-2,3-dehydroGA9 (Fig. 5, 32) was prepared by 13-deoxygenation of [1β,3-2H2]GA9 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 [1β,3-2H2]-2,3-dehydroGA9 containing 1.70 atoms of deuterium per molecule.

The stereochemistry of the deuterium labels in these substrates was determined by 2H-NMR (1). We (23) had previously established by NMR that the borodeuteride reduction of enones, e.g. [24] (Fig. 5) introduces a 1β-deuteration in the reduction products, e.g. [25] (Fig. 5). The stereochemistry of [1β,3-2H2]GA5 (Fig. 5, 29) and [1β,3-2H2]-2,3-dehydroGA9 (Fig. 5, 32) therefore follows by analogy, and the 2H-NMR spectrum (Fig. 6B) of [1β,3-2H2]GA9 methyl ester (Fig. 5, 28) showed two signals at δ2.31 and δ5.70, which can therefore be attributed to the 1β- and 3-deuterons, respectively. The assignments shown in Figure 6A can therefore be made for the 1α-, 1α-, 2-, and 3-hydrogen signals in the 1H-NMR spectrum of unlabeled GA9 methyl ester; they were confirmed by decoupling experiments. The 2H-NMR spectrum (Fig. 6C) of the prepared [1α-2H]GA5 methyl ester (Fig. 4, 22) showed one signal at δ2.56 with a possible slight shoulder at δ2.31 from which it may be conservatively concluded that the substrate [1α-2H]GA5 (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 [17-2H2]GA9 (Fig. 5, 29). The same was also the case for the 13-deoxygenation product [1β,3-2H2]-2,3-dehydroGA9 (Fig. 5, 32), since these products are not available in the literature. However, the mass spectra of the labeled compounds indicated the presence of deuterium atoms in the hydrolysates.

The presence of deuterium in the hydrolysates was confirmed by 2H-NMR, which showed the presence of deuterium in the 1,2- and 3-deuterons of the compounds. The 2H-NMR spectra of the labeled compounds showed the presence of deuterium in the 1α-, 1α-, 2-, and 3-deuterons, respectively. The assignments shown in Figure 6A can therefore be made for the 1α-, 1α-, 2-, and 3-hydrogen signals in the 1H-NMR spectrum of unlabeled GA9 methyl ester; they were confirmed by decoupling experiments. The 2H-NMR spectrum (Fig. 6C) of the prepared [1α-2H]GA5 methyl ester (Fig. 4, 22) showed one signal at δ2.56 with a possible slight shoulder at δ2.31 from which it may be conservatively concluded that the substrate [1α-2H]GA5 (Fig. 4, 23) is at least 80% stereospecifically labeled with a 1α-deuterium.

Figure 9. Metabolism of [17,13C3,2H2]GA9 by the cell-free preparation from seeds of Marah macrocarpus.

Figure 10. Metabolism of [1α-2H]GA5 by the cell-free preparation from seeds of Marah macrocarpus.
Table I. GC-MS Data

Identification of metabolites from incubations of labeled substrates with the *Marah* cell-free preparation.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolite (KRI)</th>
<th>m/z (relative intensities by GC-MS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1β,3α-H]GA₃</td>
<td>GA₃ (2525)</td>
<td>417 (M⁺, 1%), 385 (5), 357 (10), 288 (7), 282 (16), 223 (100), 207 (40), 156 (22), 75 (100), 73 (41)</td>
</tr>
<tr>
<td>[3α-H]iso GA₃</td>
<td>GA₃ (2483)</td>
<td>357 (5%), 299 (6), 281 (8), 223 (43), 208 (7), 180 (5), 156 (11), 130 (4), 113 (4), 75 (100)</td>
</tr>
<tr>
<td>[1β,3α-H]2β,3β-epoxy GA₃</td>
<td>GA₃ (2402)</td>
<td>314 (M⁺, 80%), 281 (6), 241 (95), 224 (100), 198 (27), 181 (35), 157 (47), 130 (13), 113 (28), 91 (17)</td>
</tr>
<tr>
<td>[17-²H₂]2,3-dehydro GA₃</td>
<td>GA₃ (2525)</td>
<td>418 (M⁺, 1%), 386 (1), 358 (7), 313 (5), 300 (7), 225 (37), 224 (67), 155 (19), 75 (100), 73 (42)</td>
</tr>
<tr>
<td>[17-²H₂]GA₃</td>
<td>GA₃ (2503)</td>
<td>420 (M⁺, 5%), 388 (13), 360 (2), 330 (17), 291 (31), 286 (100), 227 (59), 129 (31), 75 (28), 73 (56)</td>
</tr>
<tr>
<td>[1α-H]GA₃</td>
<td>GA₃ (2692)</td>
<td>505 (M⁺, 53%), 371 (40), 355 (20), 311 (20), 281 (13), 238 (36), 221 (16), 167 (30), 95 (90), 73 (100)</td>
</tr>
<tr>
<td>[1α-H]iso GA₃</td>
<td>GA₃ (2628)</td>
<td>505 (M⁺, 5%), 371 (6), 355 (3), 281 (5), 238 (1), 221 (1), 289 (6), 75 (100), 73 (66)</td>
</tr>
<tr>
<td>[1α-²H]1β-hydroxy GA₃</td>
<td>GA₃ (2588)</td>
<td>361 (16%), 341 (13), 282 (10), 267 (10), 217 (20), 207 (73), 183 (13), 149 (10), 109 (30), 73 (100)</td>
</tr>
<tr>
<td>[1α-²H]GA₃</td>
<td>GA₃ (2574)</td>
<td>433 (M⁺, 3%), 281 (13), 259 (3), 207 (26), 171 (13), 115 (13), 93 (10), 75 (100), 73 (43)</td>
</tr>
<tr>
<td>[17-¹³C₃-H₂]GA₃</td>
<td>GA₃ (2669)</td>
<td>505 (M⁺, 100%), 371 (37), 356 (17), 297 (14), 282 (11), 239 (31), 222 (13), 209 (13), 194 (13), 75 (49)</td>
</tr>
<tr>
<td>[17-¹³C₃]1β-hydroxy GA₃</td>
<td>GA₃ (2575)</td>
<td>505 (M⁺, 60%), 388 (56), 371 (10), 342 (16), 312 (16), 281 (23), 221 (30), 207 (50), 93 (23), 76 (13)</td>
</tr>
<tr>
<td>[17-¹³C₃]GA₃</td>
<td>GA₃ (2574)</td>
<td>433 (M⁺, 43%), 304 (46), 282 (3), 236 (10), 221 (10), 208 (36), 171 (100), 135 (10), 93 (23), 76 (13)</td>
</tr>
<tr>
<td>[17-¹³C₃-H₂]GA₃</td>
<td>GA₃ (2669)</td>
<td>507 (M⁺, 100%), 492 (10), 449 (30), 377 (13), 314 (10), 236 (10), 208 (36), 181 (13), 73 (86)</td>
</tr>
<tr>
<td>[17-¹³C₃]GA₃</td>
<td>GA₃ (2664)</td>
<td>507 (M⁺, 100%), 448 (4), 376 (61), 287 (2), 232 (2), 195 (56), 180 (5), 153 (5)</td>
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<tr>
<td>[17-¹³C₃]GA₃</td>
<td>GA₃ (2818)</td>
<td>by SICM and Rt ¹³C₃¹⁰C₁₀ = 19.9:80.1</td>
</tr>
<tr>
<td>[17-¹³C₃]GA₃</td>
<td>GA₃ (2665)</td>
<td>507 (M⁺, 43%), 431 (20), 356 (10), 269 (20), 217 (36), 208 (13), 147 (33), 129 (20), 75 (100), 73 (80)</td>
</tr>
<tr>
<td>[1β,3α-H]GA₃</td>
<td>GA₃ (2574)</td>
<td>434 (M⁺, 73%), 419 (6), 371 (16), 304 (16), 281 (6), 235 (33), 208 (23), 194 (13), 181 (6), 73 (100)</td>
</tr>
<tr>
<td>[3α-H]iso GA₃</td>
<td>GA₃ (2692)</td>
<td>505 (M⁺, 40%), 371 (40), 356 (17), 312 (20), 236 (16), 222 (10), 207 (15), 193 (10), 167 (6), 75 (100)</td>
</tr>
<tr>
<td>[3α-H]GA₃</td>
<td>GA₃ (2628)</td>
<td>505 (M⁺, 26%), 371 (40), 356 (13), 238 (10), 221 (10), 194 (10), 167 (13), 75 (100), 73 (59)</td>
</tr>
<tr>
<td>[17-²H₂]GA₃</td>
<td>GA₃ (2503)</td>
<td>420 (M⁺, 11%), 388 (13), 330 (22), 286 (79), 263 (22), 226 (100), 203 (32), 169 (25), 131 (20), 75 (60)</td>
</tr>
<tr>
<td>[17-²H₂]GA₃</td>
<td>GA₃ (2525)</td>
<td>418 (M⁺, 5%), 386 (8), 300 (17), 244 (25), 224 (92), 195 (28), 181 (30), 157 (16), 131 (11), 75 (100)</td>
</tr>
<tr>
<td>[17-²H₂]GA₃</td>
<td>GA₃ (2665)</td>
<td>508 (M⁺, 21%), 474 (3), 357 (4), 311 (10), 283 (7), 269 (9), 209 (13), 169 (13), 75 (87), 73 (100)</td>
</tr>
</tbody>
</table>
Figure 11. Metabolism of [17-13C,3H2]GA20 by the cell-free preparation from seeds of Marah macrocarpus. The broken arrow denotes a step not directly established in the present study.

13C]GA29 and [17-13C]GA8 from [17-13C,3H2]GA30, where the identification was based on GC-SIM and Rt data.

The results from the feeds of [17-2H2]GA9 (Fig. 2, [13]), [17-2H2]-2,3-dehydroGA9 (Fig. 3, [17]), and [17-13C,3H2]GA4 are shown in Figure 7. [17-2H2]GA9 was not formed from [17-2H2]-GA9 and [17-2H2]-2,3-dehydroGA9, but [17-13C,3H2]GA9 was not formed from [17-13C,3H2]GA4. The metabolism of [17-2H2]-2,3-dehydroGA9 to [17-2H2]GA7 appeared to be quantitative based on the facts that no substrate remained and no other metabolites were detected. The conversion of [17-2H2]-GA9 to [17-2H2]GA7 was low and [17-2H2]GA2 and [17-2H2]-GA4m were also formed. [17-13C,3H2]GA4 gave only [13C]GA3m.

These results establish two separate branch pathways from GA9, one to GA4 and GA3m, and the other to GA7, probably via 2,3-dehydroGA9. The conversion of 2,3-dehydroGA9 to GA7 involves the loss of one of the hydrogens at carbon-1. To determine the stereochromy of the loss, [1β,3-2H2]-2,3-dehydroGA9 (Fig. 5, [32]) was synthesized and incubated in the Marah system. As shown in Figure 8, the major products were [3α-2H]GA7 and [3α-2H]GA7; 1,2-isomeric lactone (a GC artifact from GA7); a minor product was [1β,3α-2H2]GA5, 2,3-epoxide. This result shows that the 1β-hydrogen is lost from 2,3-dehydroGA9 in the conversion to GA7.

Interestingly, the Marah system also catalyzed the metab-

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>KRI</th>
<th>2H-Atoms per Molecule*</th>
<th>m/z (Relative Intensities by GC-MS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA9</td>
<td>2525</td>
<td>ND</td>
<td>+AM01618 418(M+, 3%), 386(13), 358(12), 313(6), 264(5), 224(5), 206(7), 157(22), and 44(44)</td>
</tr>
<tr>
<td>GA9</td>
<td>2692</td>
<td>1.63</td>
<td>-AM01618 506(M+, 48%), 491(4), 391(6), 372(19), 357(10), 313(6), 238(12), 207(7), 167(6), 143(3), and 44(32)</td>
</tr>
<tr>
<td>GA5</td>
<td>2669</td>
<td>1.62</td>
<td></td>
</tr>
</tbody>
</table>

* The substrate, [1β,2β-2H2]GA4, contained 1.65 atoms 2H per molecule.
olism of GA₅, the 13-hydroxyl analog of 2,3-dehydroGA₅, to GA₅, the 13-hydroxyl analog of GA₇. Thus, [17-¹³C,²H₂]GA₄ was metabolized in high yield to [17-¹³C]GA₇ as shown in Figure 9; the only other products were trace amounts of [17-¹³C]GA₅ and [17-¹³C]-1β-hydroxyGA₇. When unlabeled GA₅ was incubated in the system, no GA₅ was formed and only unmetabolized GA₅ was detected. Thus, GA₅ does not appear to be an intermediate between GA₅ and GA₇. Like the metabolism of 2,3-dehydroGA₅ to GA₅, the metabolism of GA₅ to GA₇ occurs with the loss of the 1β-hydrogen from GA₅, as shown by the following evidence. First, [18,3,²H₂]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]GA₅ (Fig. 8). Second, [1α-²H₁]GA₅ (Fig. 4, [23]) was metabolized to [1α-²H₁]GA₅ 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 GA₅ to GA₅, GA₂₀ was not metabolized to GA₅ in the Malus system. As shown in Figure 11, incubation of [17-¹³C,²H₂]GA₂₀ gave [17-¹³C]-labeled GA₁, GA₈, GA₂₀, and GA₄₀, but no [¹³C]GA₅.

**Malus System**

As with the *Marah* system (see Fig. 7), [17-²H₂]-2,3-dehydroGA₅ was metabolized to [17-²H₂]GA₅, and [17-²H₂]-GA₅ was converted into both [17-²H₂]GA₇ and [17-²H₂]GA₇. No metabolites were detected from [17-¹³C,²H₂]GA₂₀, [17-¹³C,²H₂]GA₅ was metabolized to [17-¹³C]GA₇, but only in 0.1% yield, and to [¹³C]-1β-hydroxyGA₅ in trace amounts (0.02%).

**Fungal System**

In cultures of *G. fujikuroi*, mutant B1-4a, containing AMO 1618, [18,2β,²H₂]GA₅ was converted into [²H₂]GA₅, [²H₂]GA₆, and [²H₂]GA₇ (Table II) showing that 1,2-dehydrogenation of GA₅ to GA₇, and hence GA₇, occurs with loss of the 1α- and 2α-hydrogens.

**DISCUSSION**

The results of this study indicate that GA₅ is formed from GA₅ via 2,3-dehydroGA₅ in seeds of *Marah macrocarpus* and *Malus domestica* and that GA₅ is not a precursor of GA₅ in these systems. The absence of 2,3-dehydroGA₅ as a metabolite of GA₅ is probably due to the observed efficient conversion of 2,3-dehydroGA₅ to GA₅ by these cell-free preparations.

In *Marah* and *Malus*, the branch pathway GA₅ → 2,3-dehydroGA₅ → GA₅ parallels the corresponding 13-hydroxy branch pathway, GA₅ → GA₅ → GA₅, recently established (15) in maize shoots. Moreover, the cell-free system from *Marah* seeds catalyzed the conversion of GA₅ to GA₅ in high yield.

The stereochemistry of the conversion of 2,3-dehydroGA₅ to GA₅ and of GA₅ to GA₅ 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 GA₅ and GA₅ originates in higher plants by the loss of the 1β- and 2β-hydrogens appears to be correct. Furthermore, the biosynthesis of GA₅ in maize shoots (15) and in *Marah* seeds (this paper) and of GA₅ in *Marah* and *Malus* seeds (this paper) differs from the biosynthesis of GA₅ and GA₅ in the fungus in the following respects: in these higher plant systems, GA₅ and GA₅ are formed by separate pathways; in the fungus, GA₅ is formed from GA₅ 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 GA₅ and of 2,3-dehydroGA₅ to GA₅ are novel properties of such oxygenases. They are reminiscent of allyllic 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-dehydroGA₅ and GA₅ are deuterated at position-1. However, the epoxides do not appear to be intermediates between the 2,3-enedes and the 1-en-3-ols since GA₅ was not metabolized to GA₅ in the *Marah* system. Studies on the mechanism of the formation of GA₅ and GA₅ and 2,3-dehydroGA₅ must await purification of the enzyme(s).

**ACKNOWLEDGMENTS**

We thank Dr. M. H. Beale, University of Bristol, for the gift of [17-¹³C,²H₂]GA₅, Dr. C. R. Spray, UCLA, for help in locating and collecting of fruits of *Marah macrocarpus*, Dr. P. Hedden and his colleagues, AFRIC-IACR, Long Ashton, U.K. for their help and patience in dissecting seeds of *Malus*, and Dr. V. A. Smith for help and guidance.

**LITERATURE CITED**