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BIOSYNTHESIS OF GIBBERELLIN A_{12}-ALDEHYDE, GIBBERELLIN A_{12} AND THEIR KAURENOID PRECURSORS FROM [^{14}C]MEVALONIC ACID IN A CELL-FREE SYSTEM FROM IMMATURE SEED OF *PHASEOLUS COCCINEUS*

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Key Word Index—*Phaseolus coccineus*; Leguminosae; runner bean; seed development; biosynthesis; cell-free system; HPLC; GC/MS; gibberellin.

Abstract—A cell-free system capable of gibberellin (GA) biosynthesis has been prepared from immature seed of *Phaseolus coccineus*. This system converted [^{14}C]mevalonic acid (MVA) to *ent*-kaurene, *ent*-kaurenoic acid, *ent*-Kauradienoic acid, *ent*-7α-hydroxykaurenoic acid, *ent*-6α,7α-dihydroxykaurenoic acid, GA_{12}-aldehyde and GA_{12}. *ent*-[^{14}C]Kaurene was converted to *ent*-kaurenol, *ent*-kaurenal, *ent*-kaurenoic acid and *ent*-7α-hydroxykaurenoic acid. All identifications were by GC/MS. The pathway from MVA to GA_{12}-aldehyde in this species thus appears to be the same as that found in cell-free preparations derived from immature seed of other species.

INTRODUCTION

Ceccarelli *et al.* first showed GA biosynthesis in *Phaseolus coccineus* L. using cell-free systems prepared from suspensors of immature seeds 5–10 mm in length [1–3]. Thousands of the minute suspensors, which weigh only *ca* 200 μg each, were dissected from the seeds and homogenized to produce a cell-free system which was shown to convert MVA to GA_1, GA_3 and GA_8 via *ent*-kaurene (**1**) and *ent*-7α-hydroxykaurenoic acid (**5**). Although the usual kaurenoid intermediates were obtained, GA_{12}-aldehyde (**6**) and the C_{20}-GA intermediates between *ent*-7α-hydroxykaurenoic acid and the C_{19}-GAs were not identified. We have found that much larger quantities of a highly active cell-free preparation can conveniently be prepared from embryos of *P. coccineus* at a later stage of development when the seeds are 15–25 mm long. This system converted GA_{12}-aldehyde to GA_{53}-aldehyde, seven C_{20}-GAs and five C_{19}-GAs [4]. We now report on the early part of the GA pathway, the conversion of MVA via *ent*-kaurene to GA_{12} (**9**). All the kaurenoid intermediates predicted from previous work on GA biosynthesis in immature seeds (see refs [5, 6] for recent reviews) were obtained and identified by GC/MS.

RESULTS AND DISCUSSION

Preliminary screening experiments with small-scale (100 μl) incubations indicated MVA-metabolizing activity in the low-speed supernatant (S-1) preparations from developing *P. coccineus* seed. It was assumed that the cofactor requirements of the *Phaseolus* enzymes would be similar to those from other seed-derived cell-free systems, so ATP, PEP, Mg^{2+}, Mn^{2+} and NADPH were included in all MVA incubations. Extracts from incubations with [2-^{14}C]MVA were analysed by HPLC-radiocounting (HPLC-RC), which revealed peaks at the retention times of *ent*-kaurene (**1**), *ent*-kaurenoic acid (**4**), *ent*-7α-hydroxykaurenoic acid (**5**), *ent*-6α,7α-dihydroxykaurenoic acid (**8**), GA_{12}-aldehyde (**6**) and GA_{12} (**9**). Time-course studies showed that peaks corresponding to *ent*-kaurene, *ent*-kaurenoic acid and *ent*-7α-hydroxykaurenoic acid were present within 30 min. A longer incubation period was required before peaks corresponding to *ent*-6α,7α-dihydroxykaurenoic acid, GA_{12}-aldehyde and GA_{12} could be detected. This pattern was maintained even at high enzyme:substrate ratios.

A large-scale incubation was carried out to prepare metabolites of [^{14}C]MVA for GC/MS analysis. The S-1 preparation (250 mg, equivalent to 3.2 g of embryos, *ca* 18 seeds) was resuspended in 3 ml standard incubation medium to which (*R*)-[2-^{14}C]MVA (2.05×10^5 Bq, 104 nmol) was added. After a 2 hr incubation, an acidic ethyl acetate extract, containing 1.87×10^5 Bq of radioactivity, was recovered. HPLC-RC indicated the presence of several radioactive products, in addition to unconverted [^{14}C]MVA which constituted 59% of the recovered radioactivity (Fig. 1). GC/MS analysis of individual HPLC peaks led to the identification of *ent*-kaurene, *ent*-kaurenoic acid, *ent*-kauradienoic acid (**7**), *ent*-7α-hydroxykaurenoic acid, an unknown derivative of *ent*-7α-hydroxykaurenoic acid, *ent*-6α,7α-dihydroxykaurenoic acid, GA_{12}-aldehyde and GA_{12} (Table 1). The more polar HPLC-RC peaks (Fig. 1) were not present in sufficient quantity for GC/MS analysis. Their HPLC retention properties did not correspond to those of any of the [^{14}C]GA_{12}-aldehyde metabolites previously identified in this system [4]. The HPLC peaks with the retention times of *ent*-kaurenol (**2**) and *ent*-kaurenal (**3**) contained high levels of impurities, which precluded identification by GC/MS. For this reason, and to check that *ent*-kaurene was indeed a GA-precursor in the *Phaseolus* system, further incubations were carried out using *ent*-[^{14}C]kaurene as a substrate.

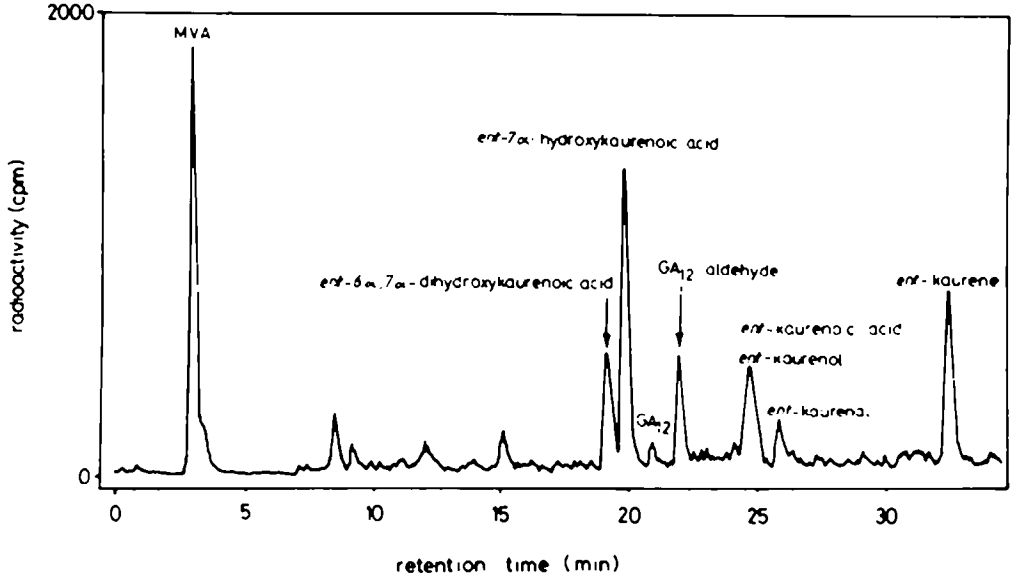

Fig. 1. Reversed-phase HPLC of products formed from [^{14}C]MVA (2.05×10^5 Bq) in a cell-free system (250 mg S-1 preparation plus co-factors) from immature seed of *P. coccineus*. HPLC conditions and detector as described in Experimental; solvent program 1.

Table 1. Summary of the principal characteristics of metabolites identified by GC/MS after incubation of [^{14}C]MVA with an S-1 preparation from immature seed of *P. coccineus*

Substance identified	*M/z* (rel. int.) [M]$^+$	[M + 8]$^+$	Other ^{14}C-ions	Specific radioactivity* (10^9 Bq mmol^{-1})
ent-Kaurene (1)	270 (62)	278 (2)		0.6
ent-Kaurenoic acid-Me (4)	316 (48)	324 (8)		1.7
ent-Kauradienoic acid-Me (7)	314 (24)	322 (5)		1.6
ent-7α-OHKA-MeTMSi (5)	404 (31)	412 (6)		1.5
ent-6α,7α-(OH)$_2$KA-Me mono-TMSi (6)	420 (10)	n.d.	[M − 15]$^+$ 405 (97), [M − 15 + 8]$^+$ 413 (3)	0.5
GA$_{12}$-aldehyde-Me (8)	330 (9)	338 (1)		0.7
GA$_{12}$-Me (9)	n.d.	n.d.	[M − 60]$^+$ 300 (100), [M − 60 + 8]$^+$ 308 (8)	1.9

*Calculated from the isotope peaks of fast, complete MS-scans.
n.d., Not detected.

Small-scale tests with the S-1 preparation using only NADPH (1 mM) as a cofactor, indicated that 70–80% of the *ent*-kaurene was metabolized within 2 hr. Although small radioactive TLC peaks corresponding to *ent*-kaurenol, *ent*-kaurenal, *ent*-7α-hydroxykaurenoic acid, *ent*-6α,7α-dihydroxykaurenoic acid, GA$_{12}$-aldehyde and GA$_{12}$ were observed, 35–50% of the radioactivity was associated with more polar compounds, thought to be other GAs and/or oxidized kaurenoid by-products. If Mn^{2+} (1 mM), which is known to inhibit the soluble oxidases involved in oxidations at C-2, C-3 and C-20 in cell-free systems from *Cucurbita maxima* [7], was also included, only 10% of the radioactivity was associated with these polar products.

In order to identify the metabolites by GC/MS, a large-scale incubation was carried out. S-1 (50 mg, equivalent to 1.03 g of embryos, *ca* three seeds) was resuspended in 3 ml of *ent*-kaurene incubation medium to which *ent*-[^{14}C]kaurene (3.33×10^4 Bq, 34.4 nmol) was added. After a 2 hr incubation, the mixture was extracted and purified by TLC-RC and HPLC-RC. Peaks with the TLC and HPLC retention properties of *ent*-kaurene (1), *ent*-kaurenol (2), *ent*-kaurenal (3), *ent*-kaurenoic acid (4) and *ent*-7α-hydroxykaurenoic acid (5) were detected. These identifications were confirmed by subsequent GC/MS (Table 2). Ions indicating ^{14}C-labelling were present in the spectra of *ent*-kaurene, *ent*-kaurenol, *ent*-kaurenal and *ent*-kaurenoic acid, but because of contaminants co-eluting on the GC, ^{14}C-labelling was not detectable in the *ent*-7α-hydroxykaurenoic acid spectrum.

Figure 2 shows that the products obtained in the incubations with [^{14}C]MVA and *ent*-[^{14}C]kaurene are identical to the intermediates in the pathway from *ent*-kaurene to GA$_{12}$ in cell-free systems from other seeds

Table 2. Summary of the principal characteristics of metabolites identified by GC/MS after incubation of *ent*-[^{14}C] kaurene with an S-1 preparation from immature seed of *P. coccineus*

Substance identified	*M/z* (rel. int.) [M]$^+$	[M + 8]$^+$	Other ^{14}C-ions	Specific radioactivity* (10^9 Bq mmol^{-1})
ent-Kaurene (substrate)	270 (69)	278 (6)		0.97
ent-Kaurene (recovered)	270 (67)	278 (4)		0.80
ent-Kaurenol-TMSi (2)	360 (7)	368 (1)	[M − 90]$^+$ 270 (96), [M − 90 + 8]$^+$ 278 (6)	0.7
ent-Kaurenal (3)	286 (97)	294 (6)		0.7
ent-Kaurenoic acid-Me (4)	316 (61)	324 (3)		0.7
ent-7α-OHKA-MeTMSi (5)	404	n.d.	[M − 90]$^+$ 314, [M − 90 + 8]$^+$ 322	n.d.

*The isotope content of *ent*-kaurene was measured by cyclic scanning of isotope peaks. Other sp. act. are calculated from fast, complete scans, which are less accurate.

n.d., Not determined.

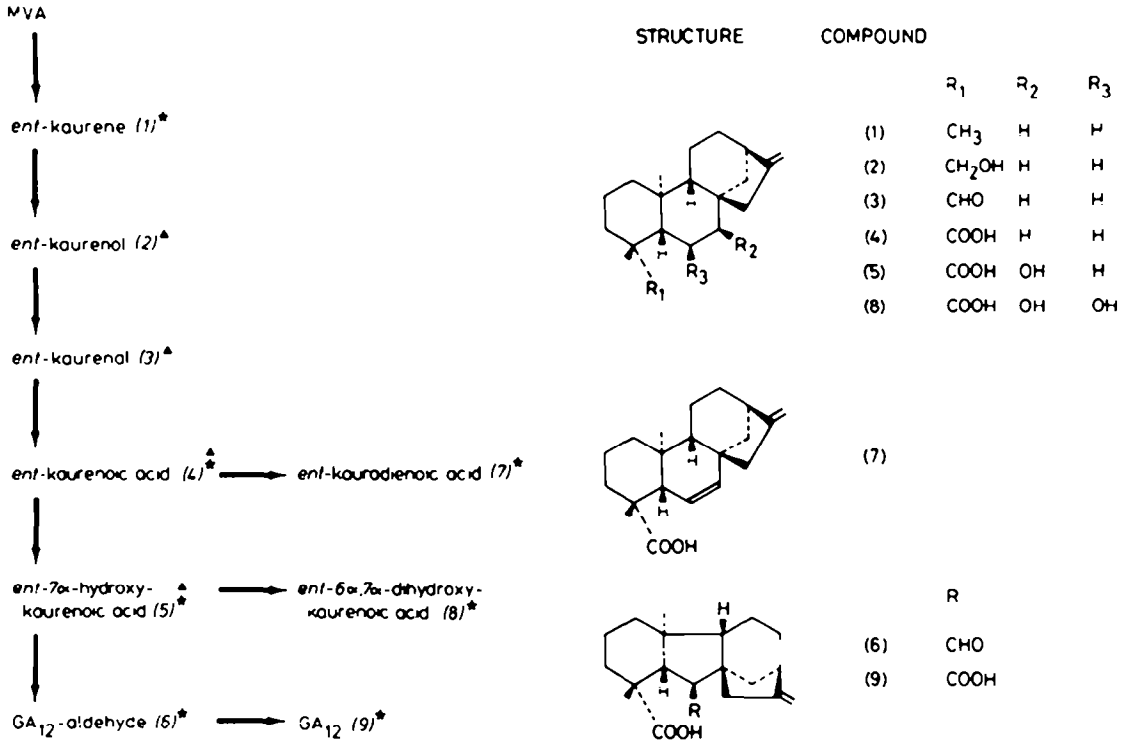

Fig. 2. Pathway of GA biosynthesis from *ent*-kaurene to GA_{12} as demonstrated in cell-free systems from immature seed of other species and in cultures of *G. fujikuroi*. ★ = identified by GC/MS as metabolite of [^{14}C]MVA in the cell-free system from *P. coccineus*. ▲ = likewise identified as metabolite of *ent*-[^{14}C]kaurene.

[5, 6] and the fungus *Gibberella fujikuroi* [8]. *ent*-Kauradienoic acid (7) is known to be the precursor of hydroxykaurenolide by-products in both *Cucurbita maxima* [9] and *G. fujikuroi* [10]. *ent*-6α,7α-Dihydroxykaurenoic acid is the first member of another branch from the GA pathway [5, 6].

Ca 170 pmol of [^{14}C]MVA were converted per mg of S-1 (protein content: 10%) during the 2 hr incubation period. Over 90% of the radioactive metabolites from [^{14}C]MVA are known members of the GA biosynthetic pathway. Thus, at least under the conditions used, MVA is preferentially converted into GAs and their kaurenoid precursors rather than into other terpenoid products.

As mentioned in the introduction, Ceccarelli *et al.* [1–3] demonstrated C_{19}-GA biosynthesis in a cell-free system from the suspensors of *P. coccineus*. Even in the young seeds used by these workers, the suspensor is very small in relation to the other seed tissues, especially the liquid endosperm present. It would therefore be extremely difficult, if not impossible, to obtain a suspensor preparation completely free of embryo and endosperm. The data presented in this paper, together with those in a previous

publication [4], demonstrate that the entire pathway of C_{19}-GA biosynthesis can be carried out in cell-free preparations derived from immature *P. coccineus* seed at a stage of development when the endosperm is degenerate and the suspensor represents only one ten-thousandth of the total fresh weight. The GA-biosynthetic capacity is thus likely to originate almost entirely from the true embryonic tissues rather than the suspensor.

EXPERIMENTAL

Preparation of cell-free system. Phaseolus coccineus L. cv. Prizewinner was field-grown in Glasgow during 1984. Pods containing immature seed were harvested from Aug. 16 to Nov. 1. The seeds were removed from the pods and grouped according to size (range of fr. wts used in present work was 350–650 mg per seed). The testas were removed and a cell-free system was prepared from remaining embryo tissues (mainly cotyledons) by grinding in ice-cold 0.05 M, K-Pi buffer, pH 7.4 (1:1, v/w) with a pestle and mortar. The homogenate was filtered through four layers of muslin, then centrifuged at 1000 *g* for 10 min at 2°. The supernatant was frozen in liquid N_2 and freeze-dried. The resultant powder, referred to as the S-1 preparation, was stored over silica gel at −20° until required.

Substrates. (*R*)-[2-^{14}C]MVA lactone (1.96×10^9 Bq/mmol) was hydrolysed with KOH to yield (*R*)-[2-^{14}C]MVA. This substrate was used directly for the production of *ent*-[^{14}C]kaurene by a cell-free system from *Cucurbita maxima* endosperm [7]. The identity and sp. act. (9.70×10^8 Bq/mmol) of the *ent*-[^{14}C]kaurene was determined by GC/MS after purification by TLC and HPLC.

Incubations. The incubation mixtures used contained anything from 0.5 to 10 mg S-1 (equivalent to 6–137 mg fr. wt of embryos) and 1.67×10^3 Bq of [^{14}C]MVA (0.85 nmol) or *ent*-[^{14}C]kaurene (1.86 nmol) resuspended in 100 μl K-Pi buffer (0.05 M, pH 7.4). Cofactors present were ATP (10 mM), PEP (10 mM), $MgCl_2$ (5 mM), $MnCl_2$ (1 mM) and NADPH (1 mM), together with bovine serum albumin (10 mg/ml). For *ent*-[^{14}C]kaurene incubations, ATP, PEP and $MgCl_2$ were omitted. The incubation mixtures were kept on ice until addition of the substrate: MVA was added in aq. soln, *ent*-kaurene was added in Me_2CO (1% of total incubation vol.). After mixing, tubes were incubated in a shaking water bath at either 28° or 30° for up to 2 hr.

Extraction. At the end of the incubation period, mixtures were acidified to pH 3 with HOAc, and Me_2CO was added to give a final concn of 80% which ppted salts and proteins. After centrifugation (1000 *g*, 5 min) and decanting the supernatant, the pellet was washed with EtOAc and re-centrifuged. The EtOAc was added to the first supernatant followed by 1/3 vol. dilute HOAc (pH 3). After mixing, the extract was centrifuged and the organic phase removed. The aq. phase was extracted × 4 more with an equal vol. of EtOAc. The organic fractions were combined, washed with 1/4 vol. of 1% HOAc then evaporated to dryness under N_2 or by rotary evaporation at 40°.

TLC. (a) Preparative MVA incubation: the EtOAc extract was run on a silica gel plate developed in toluene–EtOAc (19:1), then examined with a radiochromatogram scanner. The *ent*-kaurene peak at the solvent front was eluted separately. The rest of the radioactivity was between the origin and R_f 0.35 and was eluted as one fraction.

(b) *ent*-Kaurene incubations: extracts were run on silica gel plates developed in petrol (boiling range 60–80°), then re-developed to 13.5 cm in $CHCl_3$–EtOAc–HOAc (75:25:1), followed by TLC-RC.

HPLC. (a) MVA incubations: A Spectra Physics SP8700 solvent delivery system was used (Spectra Physics, St. Albans, U.K.). Samples were introduced off-column via a Rheodyne 7125 sample injection value fitted with a 200 μl sample loop. Reversed-phase separations were carried out on a 250 × 5 mm i.d. column packed with 5 μm ODS Hypersil (Shandon, Runcorn, England) eluted with mixtures of MeOH and dilute aq. HOAc (pH 3). Three solvent programs were used (1) 0–15 min, 50–80% MeOH; 15–19 min, 80–100% MeOH; 19–44 min, 100% MeOH; (2) isocratic 100% MeOH; (3) 0–12 min, 80% MeOH; 12–13 min, 80–100% MeOH; 12–33 min, 100% MeOH. For analytical runs, column eluate (1 ml/min) was mixed with scintillant (3 ml/min) and passed through a spiral glass flow-cell (300 μl) in a Coruflow manual scintillation counter. The scintillant consisted of 10 g/l 2,5-diphenyloxazole in Triton X-100–xylene–MeOH (11:22:5). Counting efficiency was *ca* 60% for ^{14}C. For preparative runs of TLC-purified samples, fractions (0.5 or 1.0 ml) were collected directly and radioactivity determined by liquid scintillation counting of suitable aliquots. Program (1) was used for all analytical work and for initial separation of preparative extract. *ent*-Kaurene was rechromatographed by program (2), and all other fractions by program (3).

(b) *ent*-Kaurene incubations. A Waters 6000 A pump system coupled to an M-45 automated gradient controller was used. Samples were introduced via a Rheodyne 7125 injector fitted with a 100 μl sample loop. Reversed-phase separations were carried out on a 120 × 8 mm i.d. radial compression cartridge (C18 Novapak 5 μm) eluted with mixtures of redistilled MeOH and 0.01 M HOAc (pH 3.2). Three gradients were used: (4) 0–10 min, 90–100% MeOH; 10–40 min, 100% MeOH; (5) 0–20 min, 85–100% MeOH; 20–35 min, 100% MeOH; (6) 0–30 min, 50–100% MeOH; 30–38 min, 100% MeOH. Column eluant was passed through a RamonA Raytest (Isomess) radioactivity monitor fitted with an Yttrium silicate solid scintillant flow-cell (^{14}C counting efficiency, 15%) and fractions were collected according to peaks seen on the monitor read-out. Program (4) was used for *ent*-kaurene and *ent*-kaurenal peaks; program (5) for *ent*-kaurenol and *ent*-kaurenoic acid; program (6) for *ent*-7α-hydroxykaurenoic acid.

Derivatization. Dry samples were dissolved in MeOH and methylated with CH_2N_2 in Et_2O. The methylated extracts were dried and then silylated in sealed tubes by treatment with 2 μl of N,O-bis-(trimethylsilyl)trifluoroacetamide or N-methyl-N-trimethylsilyltrifluoroacetamide at 60° for 50 min.

GC/MS. GC/MS was carried out using a Finnigan 4015 GC/MS/data system. Derivatized samples were injected (260°) in 1 μl aliquots into a fused silica capillary column (SE-30 chemical bonded phase, 25 m × 0.32 mm) using a Grob splitless injector. The column temperature was maintained at 50° for 1 min, then programmed at 15°/min to 200° and at 4°/min to 260°. The He flow rate was 2 ml/min. The split was opened 1 min after injection. The column effluent was led directly into the ion source at 260°. Electron energy was 30 eV and emission current was 0.24 mA.

Reference compounds and mass spectra. All metabolites were identified by comparison of their mass spectra with those of authentic compounds. Isotope contents were determined from the mass spectrum by the method of ref. [11]. Since the main purpose was identification, complete spectra were usually recorded. The specific activities in most cases were therefore calculated from single, fast scans, which show considerable statistical variability [12]. The two values for *ent*-kaurene in Table 2 were obtained by cyclic scanning over the M + isotopic peaks, and therefore are more reliable.

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