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GIBBERELLIN BIOSYNTHESIS IN A CELL-FREE SYSTEM FROM IMMATURE SEEDS OF PISUM SATIVUM[1]

Hans-Joachim Ropers[2] and Jan E. Graebe

Pflanzenphysiologisches Institut der Universität
Untere Karspüle 2, 3400 Göttingen
Federal Republic of Germany

Paul Gaskin and Jake MacMillan

School of Chemistry, The University of Bristol
Bristol BS8 1TS, U.K.

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Summary: A cell-free system from immature pea seeds converts ^{14}C-labelled ent-kaurene to ent-kaurenol, ent-kaurenal, ent-kaurenoic acid, ent-7α-hydroxykaurenoic acid, and gibberellin A_{12}-aldehyde. The latter becomes converted further to 13-hydroxygibberellin A_{12}, gibberellin A_{44}, gibberellin A_{12}-alcohol, and several unidentified products. Thus the biosynthesis of gibberellins via ent-kaurene is now established for a member of the Leguminosae. It is the first time that 13-hydroxylation of gibberellins has been observed in a cell-free system and that gibberellin A_{12}-alcohol has been obtained in any biological system.

The part of the GA[3] biosynthetic pathway shown in Scheme 1 has previously been established in the ascomycete *Gibberella fujikuroi* and in cell-free systems of two species belonging to *Cucurbitaceae*, *Marah macrocarpus* and *Cucurbita maxima*. The last product of the sequence, GA_{12}-aldehyde (VI), is a precursor of C_{20}- and C_{19}-GAs in *G. fujikuroi* and *C. maxima* but the pathway by which it becomes converted to C_{19}-GAs has not been fully

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[3] Abbreviations: GA, gibberellin; GC-MS, combined gas chromatography-mass spectrometry.

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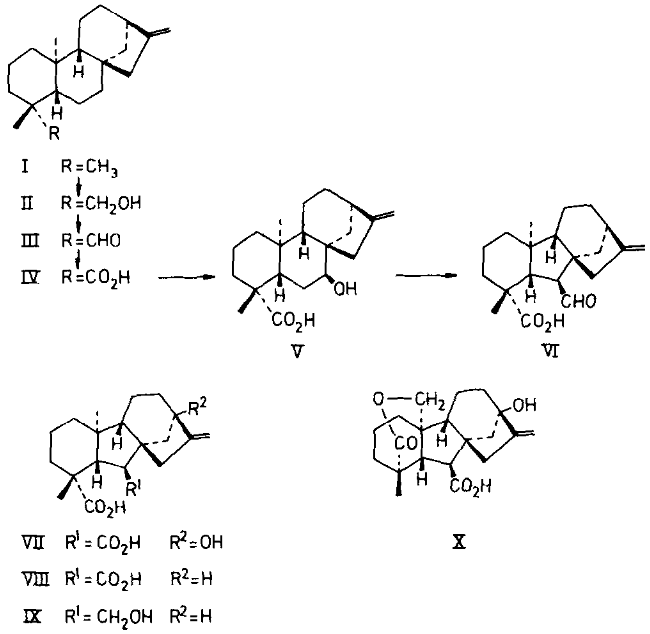

Scheme 1. Conversion of *ent*-kaurene (I) to GA$_{12}$-aldehyde (VI) *via* *ent*-kaurenol (II), *ent*-kaurenal (III), *ent*-kaurenoic acid (IV), and *ent*-7α-OH-kaurenoic acid (V).

elucidated (1).

Cell-free systems from immature pea seeds produce *ent*-kaurene (I) from mevalonate but the conversion is very low (2,3). Small amounts of the second member of the sequence, *ent*-kaurenol (II), may also be formed with mevalonate as the substrate (4), but direct metabolism of *ent*-kaurene to defined products could not be demonstrated (4,5). In spite of this, the pea seed appeared a promising object for the study of GA biosynthesis because it produces large amounts of mainly 13-hydroxylated C$_{19}$-GAs at defined stages of development (6,7). A cell-free system could be expected to yield information on GA biosynthesis in general and on the 13-hydroxylation step in particular.

MATERIALS AND METHODS

Immature seeds (approaching full size) of field-grown Pisum sativum L., cv. "Großer Schnabel mit Gedrücktem Korn" were homogenized with phosphate buffer (0.05 M, pH 8.0, 1:1, w/v). The homogenate was filtered and centrifuged at 2 000 x g for 5 min or at 200 000 x g for 1 h. The supernatant was stored in liquid N_2 until used as enzyme preparation.

Substrates were generated from [2-^{14}C]mevalonate with endosperm preparations from Cucurbita maxima (8). They were purified by TLC and their identities were confirmed by GC-MS.

The incubation mixtures are described in Table 1 and Fig. 1. After incubation, pH was adjusted to 3.0, acetone was added and the incubation products were extracted with EtOAc. TLC was on Al-sheets precoated with silica gel 60 and Kieselgur F254 (1:1). The solvent systems were: 1) $CHCl_3$/EtOAc/HOAc = 40:60:1; 2) $CHCl_3$/EtOAc/HOAc = 50:50:1, developed 2x in the same direction; 3) petroleum ether (40°- 60°)/acetone/isobutyric acid = 90:10:1, developed 3x; 4) benzene to 15 cm - the peaks were then located by scanning and the upper 7.5-8.0 cm (containing ent-kaurene and ent-kaurenal unresolved) were re-developed with petroleum ether to the edge after having been cut off from the rest of the sheet.

Radioactive fractions were eluted from the gel, methylated and silylated, and analyzed by GC-MS on 2% SE-33 columns. Fractions containing GA_{12}-aldehyde were additionally analyzed on QF-1 columns to separate GA_{12}-aldehyde from GA_{12}. Spectra were recorded on a DEC Linc 8 computer. All metabolites were identified by full m.s. scans. Specific radioactivities were determined from the mass spectra obtained by single scans and are not accurate (9).

RESULTS

When ent- [^{14}C]kaurene (I) was incubated with the cell-free system from immature pea seeds as described in Table 1 several products resulted, which were separated by TLC and identified by GC-MS as ent-[^{14}C]kaurenol (II), ent-[^{14}C]kaurenal (III), ent-[^{14}C]kaurenoic acid (IV), ent-7α-hydroxy-[^{14}C]kaurenoic acid (V), and [^{14}C]GA_{12}-aldehyde (VI) (Table 1). Table 2 shows that reincubation of each product confirmed the biosynthetic sequence shown in Scheme 1. This series of conversions needed mainly NADPH as a cofactor. Addition of ATP, P-enolpyruvate, and Mg^{2+} only increased the conversion of ent-kaurene by about 10% and increased the proportions of products at the far end of the sequence. No conversion of ent-kaurene occurred with boiled enzyme.

TABLE 1. IDENTIFICATION OF PRODUCTS OBTAINED BY INCUBATION OF PEA SEED ENZYME SYSTEM WITH *ent*-[^{14}C]KAURENE

R_f [a]	dpm [b]	Major ^{14}C-component by GC-MS	Spec. act. [c] (mCi/mmol)
0.00	1061 x 10^3	unidentified	
0.00	215 x 10^3	*ent*-7α-OHKA (V)	44
0.03	199 x 10^3	GA_{12}-aldehyde (VI)	38
		GA_{12} (VIII)	41
0.09	980 x 10^3	*ent*-kaurenoic acid (IV)	40
0.26	1905 x 10^3	*ent*-kaurenol (II)	41
0.73	3718 x 10^3	*ent*-kaurenal (III)	39
0.93	6118 x 10^3	*ent*-kaurene (I)	38

The incubation mixture consisted of $MgCl_2$ (10 m*M*), ATP (10 m*M*), P-enolpyruvate (20 m*M*), NADPH (1 m*M*), *ent*-[^{14}C]-kaurene (25.9×10^6 dpm, 41 mCi/mmol) and 2 000 *g* supernatant preparation (18.7 ml) in a final volume of 20.9 ml. Incubation was for 4 h at 30°.

[a] On initial TLC in solvent system 4.

[b] After purification in several solvent systems.

[c] Approximations only as determined from single scan mass spectra.

When [^{14}C]GA_{12}-aldehyde (VI) was incubated with low speed (2 000 *g*) supernatant and the several products were separated by TLC, the pattern shown in Fig. 1*a* resulted. The major product (R_f 0.41) was separated further by TLC system 2 into a major fraction (2.4×10^6 dpm), which contained 13-OH-[^{14}C]GA_{12} (VII) as a main component, and a minor fraction (4.5×10^5 dpm), which contained [^{14}C]GA_{44} (X). Both products were identified by GC-MS. The fraction at R_f 0.86 (Fig. 1*a*) after purification

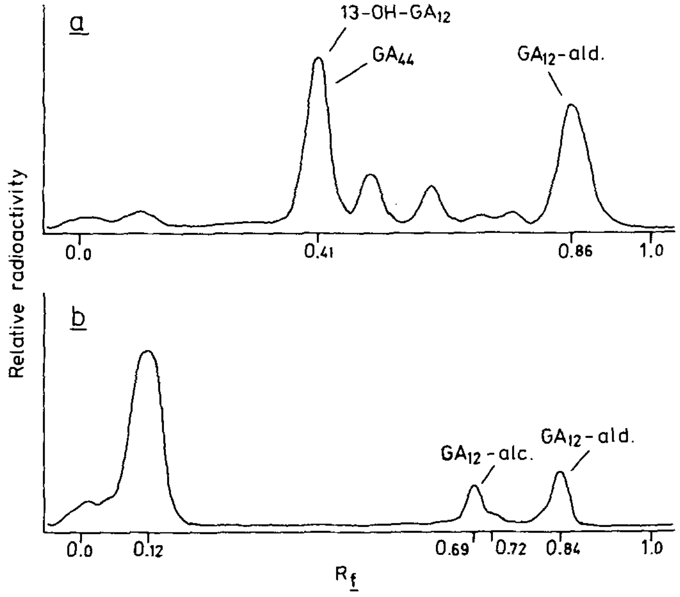

Fig. 1. Radiochromatograms of extracts obtained from incubations of a) low speed (2 000 g) or b) high speed (200 000 g) supernatant preparations with [^{14}C]GA$_{12}$-aldehyde.

Cofactors as in Table 1 plus FeSO$_4$ (0.5 mM) were incubated with a) 2 000 g supernatant (10.2 ml) and [^{14}C]GA$_{12}$-aldehyde (17.3 x 10^6 dpm) in a total volume of 12 ml or b) 200 000 g supernatant (8.5 ml) and [^{14}C]GA$_{12}$-aldehyde (11.1 x 10^6 dpm) in a total volume of 10.0 ml at 30° for 4 h. Separation of products in solvent system 1.

in several solvent systems contained 2.4 x 10^6 dpm. It was identified by GC-MS as a mixture of [^{14}C]GA$_{12}$-aldehyde (VI) and [^{14}C]GA$_{12}$ (VIII). The presence of the latter compound was an artefact, since according to the TLC record, it was not originally a component of this fraction.

Unlike the conversion of ent-kaurene, the conversion of GA$_{12}$-aldehyde was not dependent on NADPH, but it was dependent on Fe^{2+} and ATP. Thus, the conversion was only 50% of its maximal rate when Fe^{2+} was omitted from the incubation mixture as specified in Fig. 1, and it was only 15% of the maximal

TABLE 2. DEMONSTRATION OF INTERMEDIACY OF METABOLITES IN THE CONVERSION OF ent-KAURENE TO GA_{12}-ALDEHYDE

Substrate[a]	^{14}C-Substances identified[b]					
	K-ene	K-ol	K-al	KA	OHKA	GA_{12}-ald
ent-[^{14}C]Kaurene	+	+	+	+	+	+
ent-[^{14}C]Kaurenol	0	+	+	+	+	+
ent-[^{14}C]Kaurenal	0	0	+	+	+	+
ent-[^{14}C]Kaurenoic acid	0	0	0	+	+	+
ent-7α-OH-[^{14}C]kaurenoic acid	0	0	0	0	+	+

[a] The substrates indicated were incubated with the pea system as in Table 1.

[b] The products of the kaurenol- and kaurenal-incubations were identified by TLC. All other products were purified by TLC and identified by GC-MS, which also showed that the spec. radioactivity of substrates and products closely agreed.

rate when Fe^{2+}, ATP and P-enolpyruvate were omitted.

The product pattern shown in Fig. 1a was dependent on particulate matter in the enzyme preparation. When [^{14}C]GA_{12}-aldehyde (VI) was incubated with high speed (200 000 g) supernatant system, the pattern shown in Fig. 1b resulted. The major product (R_f 0.12, 5.3×10^6 dpm) could not be identified by GC-MS because it decomposed on the column during GC. The double peak at R_f 0.69 - 0.72 was resolved into two clear fractions by solvent system 3. The major one of these (420 000 dpm) consisted almost entirely of [^{14}C]GA_{12}-alcohol (IX) by GC-MS. The minor fraction could not be identified. The material at

R_f 0.84 (Fig. 1b, 1.4 x 10^6 dpm) was identified by GC-MS as residual [^{14}C]GA_{12}-aldehyde (VI).

When the unidentified material at R_f 0.12 (Fig.1b) was incubated with low speed supernatant system, it was converted very efficiently to several components, most of which were less polar than the substrate. Like the substrate, these products decomposed on GC and therefore could not be identified.

DISCUSSION

The results demonstrate the sequence shown in Scheme 1 in the cell-free system from pea seeds. Although some of the products were obtained in small amounts, the over-all conversion was considerable: 53% of the recovered label, representing 27% of label added, was associated with conclusively identified products.

13-HydroxyGA_{12} (VII) is a major product of GA_{12}-aldehyde (VI) in the system. This and the formation of GA_{44} (X) shows that GA biosynthesis in the cell-free system is closely related to that of intact seeds. In these all the major GAs are 13-hydroxylated, GA_{44} (X) being one of them (6,7). The results do not show whether 13-hydroxyGA_{12} is an intermediate in the biosynthesis of pea GAs, but they do support the view (10,11) that 13-hydroxylation is an early event in the pathway.

The production of GA_{12}-alcohol (IX) from GA_{12}-aldehyde (VI) is surprizing. GA_{12}-alcohol is a very efficient precursor of GA_3 in G. fujikuroi but no evidence has been found for its participation as a natural intermediate of GA biosynthesis in that organism (12-15). This is the first time that GA_{12}-alcohol has been obtained in a biological system.

The pea system differs from existing systems of GA biosynthesis by the very active 13-hydroxylation and by the formation of metabolites with properties distinct from those of known GA intermediates. The system certainly will be useful for the study of 13-hydroxylation. Whether it will be useful for the study of C_{19}-GA biosynthesis will depend on the identities of the unidentified metabolites. The very high yields in which they are formed indicate significance. Furthermore, the fact that one of them is converted to less polar products in the mainly oxidative pathway is promising, since this is what would be expected when C_{20}-GAs are converted to C_{19}-GAs.

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