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GIBBERELLIN METABOLISM

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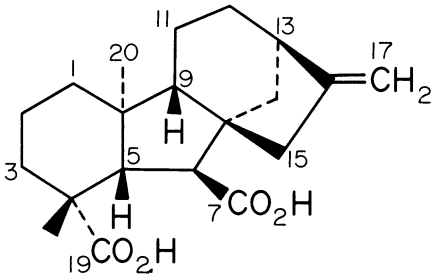
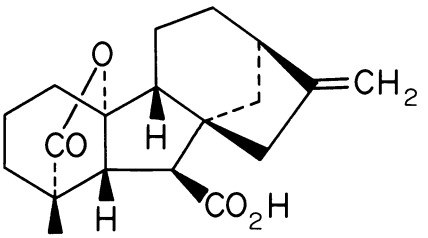
Abstract - Considerable progress has been made in the last few years in determining the biosynthetic pathways to the gibberellins in cultures of Gibberella fujikuroi and cell-free systems from the endosperm of Cucurbita maxima and Pisum sativum. Metabolic studies with intact higher plants are fraught with technical difficulties. The use of [^{2}H]GAs together with mass spectrometry offers a useful approach towards the solution of some of these problems.

INTRODUCTION

It is a particular pleasure to return to the birthplace of the gibberellins and describe some aspects of our recent work. Since their discovery early in this century as plant-growth promoting metabolites of the rice pathogen, Gibberella fujikuroi, the gibberellins have grown in stature. They are now known to be of widespread, and possibly universal, occurrence in green plants in which they are believed to function as hormones regulating growth and development. To date 52 gibberellins have been isolated: 15 from the fungus; 30 from higher plants; and 7 from both the fungus and higher plants. The structures of these gibberellins are oxidative variants of the two basic structures shown with numbering in Fig. 1. For convenience gibberellins (GAs) are allocated trivial names in the series GA_1 to GA_n (Ref. 1).



C_{20}-gibberellin



C_{19}-gibberellin

Fig. 1 GA-nomenclature

The metabolism of the GAs has special importance in relation to their hormonal function. A main goal is to provide information on the biosynthesis and deactivation of the GAs and hence on the regulation of the endogenous levels of the active hormones. To do so in developing intact plants poses formidable problems. In addition to the difficulties

associated with the low levels of GAs present in higher plants and the low incorporation of precursors, special problems exist in designing feeding experiments to examine GA-metabolism in relation to the developmental processes which the GAs may be regulating. Some of these problems are discussed later. But first a brief review is provided of the substantial progress that has been made in elucidating GA-biosynthesis using cultures and cell-free systems from G. fujikuroi and in cell-free systems from the endosperm of Marah macrocapus (wild cucumber), Cucurbita maxima (pumpkin), and Pisum sativum (pea).

BIOSYNTHESIS IN G. fujikuroi AND HIGHER PLANT CELL-FREE SYSTEMS

Formation of the ent-gibberellane ring system

In all of the systems so far studied in detail, the pathway (Fig. 2) from MVA to GA_{12}-aldehyde is the same. This pathway may therefore be common to all higher plants. The interesting ring B contraction has been examined recently by Graebe et al. (2, and unpublished results) and shown to involve loss of the ent-6α-hydrogen and retention of the ent-6β and ent-7β-hydrogens in going from ent-7α-hydroxykaurenoic acid to GA_{12}-aldehyde and to ent-6β,7α-dihydroxykaurenoic acid.

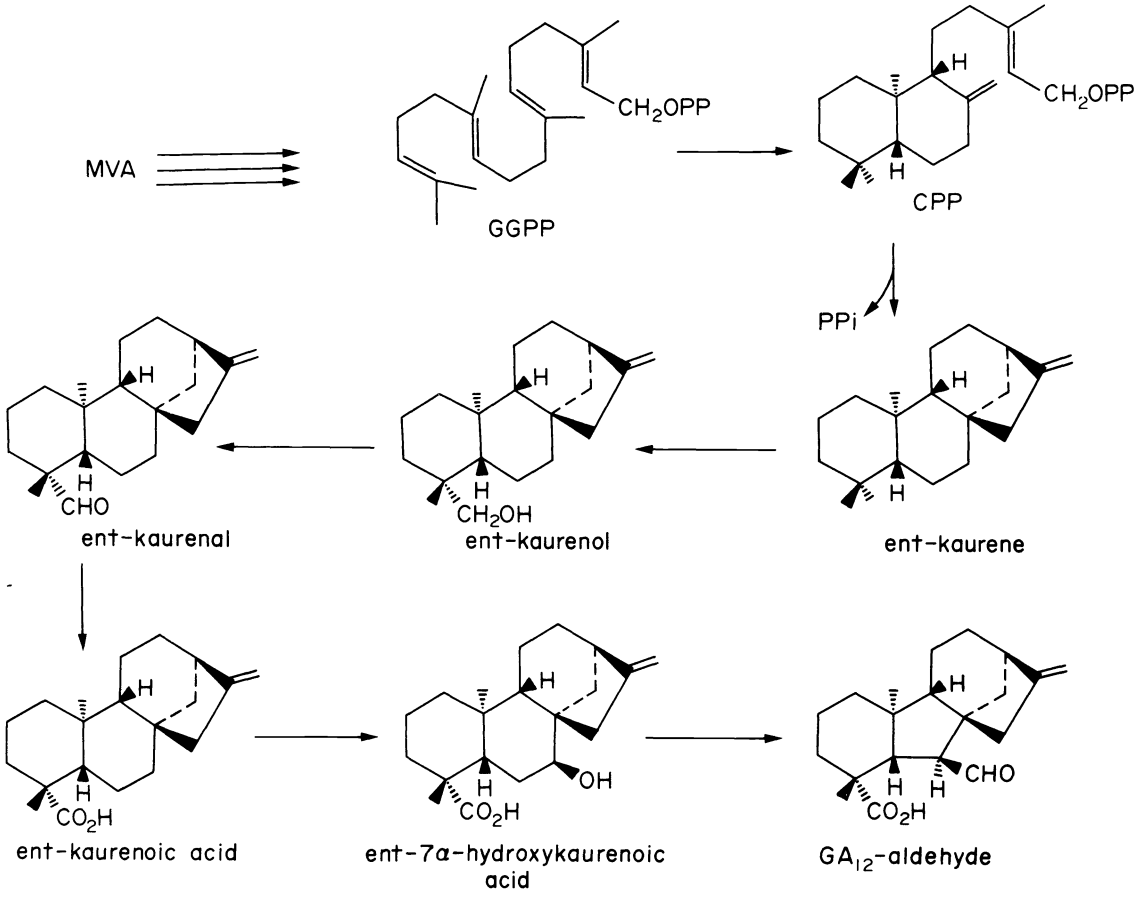


Fig. 2 Pathway from MVA to GA_{12}-aldehyde

Pathways from GA_{12}-aldehyde

From GA_{12}-aldehyde the pathways differ for each system depending upon the order in which oxidation occurs.

In the G. fujikuroi, there are two pathways which branch from GA_{12}-aldehyde (Fig. 3). In the main pathway to GA_3, 3β-hydroxylation is the first step and 13-hydroxylation is the last step. The minor pathway to GA_9 involves no hydroxylation although 13-hydroxylation of GA_9 does occur to a small extent. 2α-Hydroxylation of GA_4 and GA_9 and 1α-hydroxylation of GA_4 is also observed to a small extent. 2β-Hydroxylation is absent in the fungus. The enzymes which catalyse GA-biosynthesis in the fungus have apparently low substrate specificity and will convert analogues of the natural intermediates into analogues of the fungal GAs, some of which are higher plant GAs (see, for example, Ref. 3 and 4).

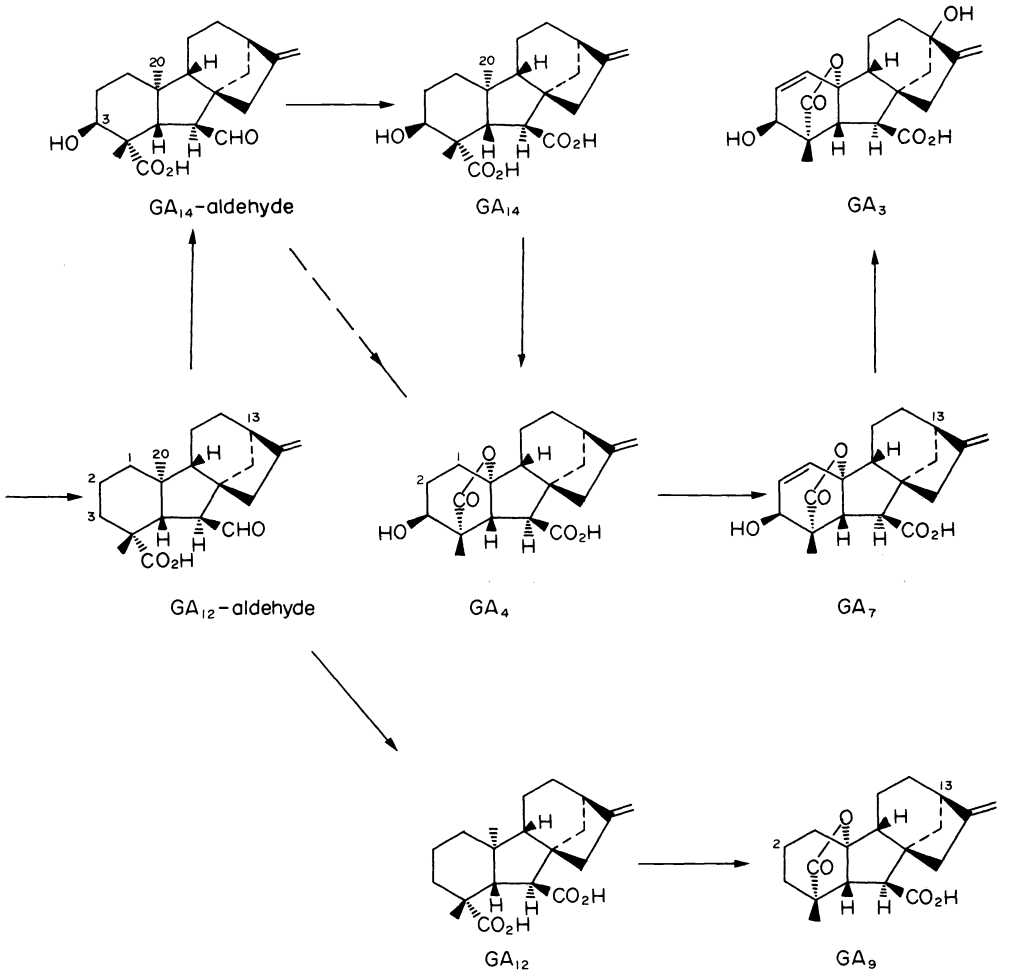


Fig. 3 G. fujikuroi: simplified pathway from GA_{12}-aldehyde

In the C. maxima preparations an S_{200}-fraction which requires Fe^{2+} converts GA_{12}-aldehyde into GA_4 and GA_{43} (Fig. 4) (Ref. 5 and 6). Since GA_{43} occurs in the endosperm and GA_4 occurs in the embryo of C. maxima seeds these pathways probably operate in vivo. 3β-Hydroxylation which is catalysed by a microsomal fraction, takes place later in the main pathway to GA_{43}. In contrast to the fungal pathways 2α- and 13-hydroxylation have not been observed and 2β-hydroxylation occurs. Alternative pathways to GA_{43}, including 3β-hydroxylation of GA_{12}-aldehyde, can however operate when high substrate levels are used. Since enzymes cannot be induced in a cell-free system, these alternative pathways, point to low substrate specificity of the enzymes in higher plants as well as in the fungus, G. fujikuroi.

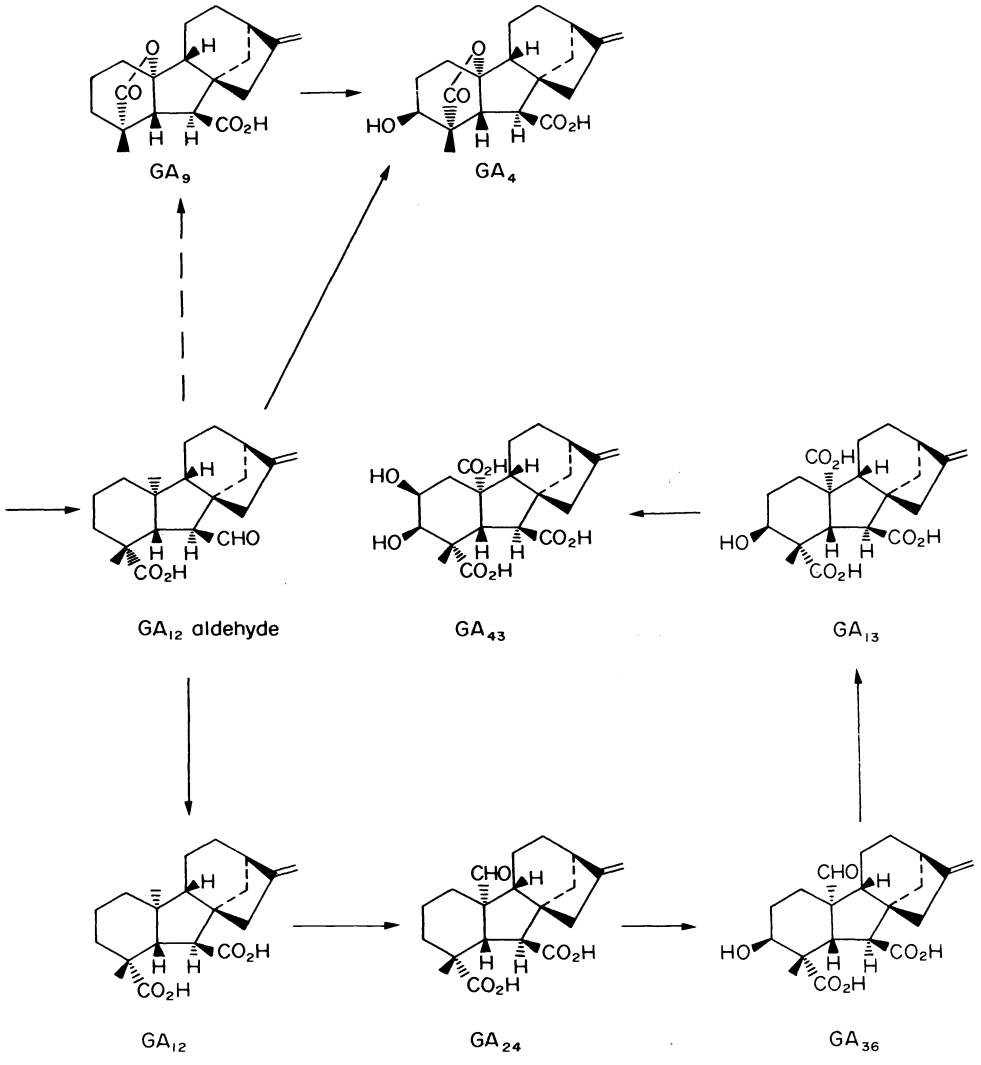


Fig. 4 C. maxima: simplified pathway from GA_{12}-aldehyde

In the P. sativum preparations (Fig. 5) a particulate fraction converts GA_{12}-aldehyde to 13-hydroxy GA_{12} together with smaller amounts of 13-hydroxy GA_{12}-aldehyde and GA_{44} (Graebe, Ropers, Sponsel and MacMillan, unpublished results). The last mentioned compound has been identified in seed of P. sativum (Ref. 7). Thus it appears that 13-hydroxylation occurs immediately after GA_{12}-aldehyde in contrast to (a) the fungus where 13-hydroxylation is the last step and (b) the C. maxima system where 13-hydroxylation is absent. Further steps in the pathway in P. sativum preparations are being investigated. However, further information on later steps has been obtained from studies with intact seeds of P. sativum and is discussed later.

Conversion of C_{20}-GAs to C_{19}-GAs

The mechanism by which carbon-20 is removed in this conversion is a major remaining problem. No intermediates are known between C_{20}-GAs with a 10-methyl group and the C_{19}-GAs. Recently Bearder et al.(8) have shown by ^{18}O-labelling experiments that both oxygens of the lactone

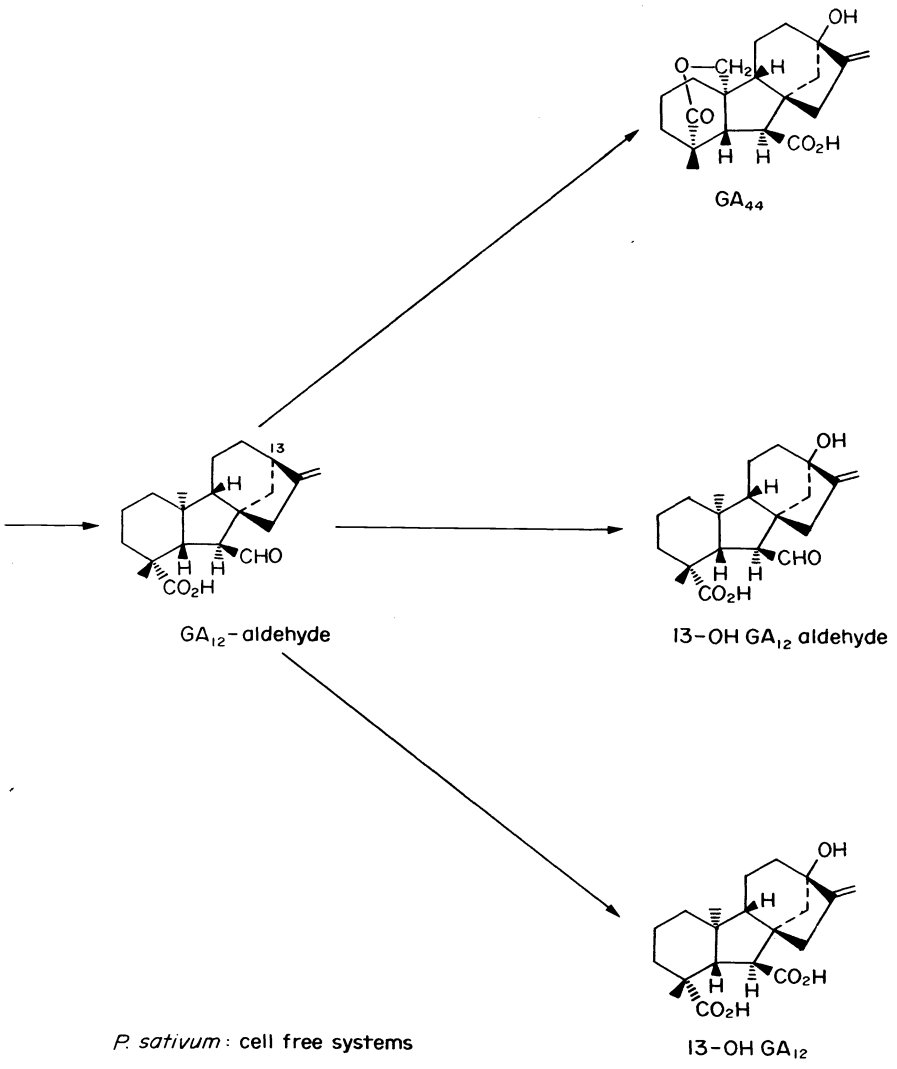


Fig. 5 P. sativum: cell-free system

bridge in the fungal C_{19}-GAs are derived from the 19-oic acid of their C_{20}-GA precursors. This finding limits the possible mechanism but the oxidation level at which carbon-20 is lost is still unknown.

METABOLIC STUDIES IN INTACT PLANTS

In intact plants, the pathway to GA_{12}-aldehyde (Fig. 2) has not been firmly established and none of the intermediates up to and including GA_{12}-aldehyde have been shown to be incorporated to positively identified GAs. Consequently most investigations have concentrated on the metabolism of the GAs themselves.

To determine the metabolism of arbitrarily selected GAs, fed to intact plants at arbitrarily selected stages of development has intrinsic enzymological interest. However to correlate GA metabolism with development in growing plants, and hence with hormonal function, imposes severe restraints. In our studies using developing seed of P. sativum cv. Progress No. 9 (Ref. 7,9,10,11) we have tried to apply the following criteria to establish a metabolic step, GA_x to GA_y, as a normal process: (a) GA_x and GA_y should be native to the system otherwise metabolism of the added GA_x may simply be non-specific due to low substrate

specificity of the plant enzymes; (b) labelled GA_x should be fed at a stage of development when native GA_x is known to be present in the plant material; (c) labelled GA_x should be fed at a concentration close to that known to be native to the plant system at the time of feeding; and (d) the formation of labelled GA_y should coincide with the formation of the native GA_y. To meet these criteria the native GAs were identified and quantified at eleven stages during seed development (Ref. 9,10). The sequential rise and fall of GA_9, GA_{20} and GA_{29} suggested the sequence $GA_9 \rightarrow GA_{20} \rightarrow GA_{29}$ (see Fig. 6 for structures). When $[^3H]GA_{20}$ was fed under conditions which satisfied the above criteria $[^3H]GA_{29}$ was formed in yields up to 90% and it seems reasonable to conclude that the conversion of GA_{20} to GA_{29} is a normal metabolic process in developing seed of P. sativum cv. Progress No. 9. The levels of the native GA_9 were too low to meet criterion (c). In order to identify the metabolites 10-50 times the normal concentration had to be used. However when $[^3H]GA_9$ was fed under conditions which satisfied the other criteria $[^3H]GA_{20}$ was not produced. Instead $[^3H]GA_{51}$ was formed and subsequently shown to be native to seed at the appropriate developmental stage. Two other metabolites which were tentatively identified as 12α-hydroxy $[^3H]GA_9$ and its conjugate, could not be detected as native compounds and may be artefacts formed as a result of feeding more than the natural level of GA_9. When $[^3H]GA_9$ was fed to seed at an earlier stage of development when neither GA_9 or GA_{20} were present as native GAs, it was metabolised to $[^3H]GA_{20}$ and not $[^3H]GA_{51}$. The tentatively identified 12α-hydroxy $[^3H]GA_9$ and its conjugate were also formed. From these results it is reasonable to conclude that GA_9 is the natural precursor of GA_{51} and not of GA_{20}. Gibberellins A_{20} and A_9 are probably formed by independent pathways, presumably from GA_{12}-aldehyde (Fig. 6). In view of the evidence, presented earlier, for a 13-hydroxylation step immediately after GA_{12}-aldehyde in cell-free preparations from pea seed, the pathway to GA_{20} probably involves an early 13-hydroxylated intermediate. Thus the conversion of $[^3H]GA_9$ to $[^3H]GA_{20}$ in younger seed may simply be the non-specific 13-hydroxylation by a 13-hydroxylase of low specificity. This enzyme activity is no longer present when the seed begins to synthesise the native GA_9. This line of thought suggests that the oxygenation sequence may be regulated by the appearance and disappearance of 13- and 2β-hydroxylase activity.

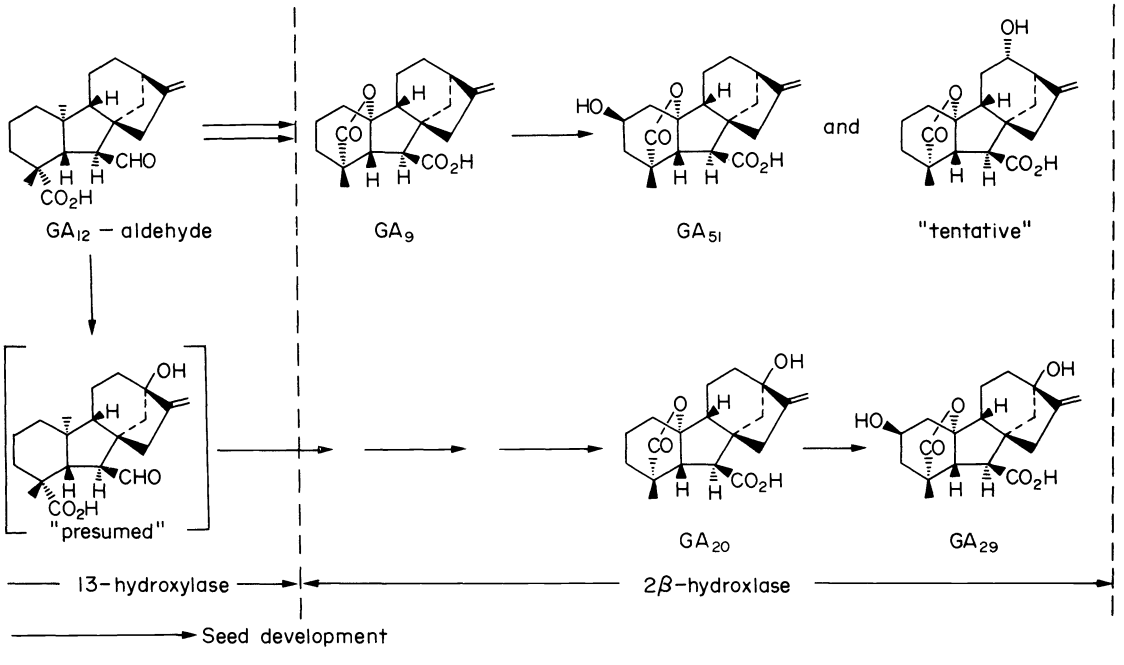


Fig. 6 Proposed metabolic steps in developing seed of P. sativum cv. Progress No. 9

Two further problems in establishing a metabolic step, $GA_x \rightarrow GA_y$ are (a) the difficulty in identifying the metabolite GA_y in the presence of the native GA_y; for example in the work just described, the metabolites were identified by the negative evidence that the radioactive metabolite had the correct R_T by GC-RC and sufficient mass to show the absence of other compounds at the correct R_T by GC-MS, and (b) the problem of establishing that the labelled GA_x which is fed is equivalent to the native GA_x. Both points can, in principle, be solved using stable isotopes. For this purpose we have recently synthesised several [^{3}H]GAs from the commercially available fungal GA_3 and GA_7 (unpublished results). One of the synthetic routes (Fig. 7) is based upon the hydride reduction of the enones derived from GA_3 and GA_7 methyl esters by MnO_2 oxidation (cf. Ref. 12). The mechanism of the reduction of enones to the saturated alcohols has not been explicitly discussed in the literature. It requires a proton source. Using NaB^xH_4 (x = 1,2 or 3), LiBr and diglyme we find that the proton source is adventitious water in the solvent as previously suggested (Ref. 13,14). However in anhydrous tetrahydrofuran, we find that the reduction is a two-step process (Fig. 7). During acidic work-up the initially formed Li enolate of the saturated ketone is converted to the saturated ketone faster than the decomposition of the $B^xH_4^-$ (cf. Ref. 15) which then reduces the saturated ketone to the saturated alcohol. The proton (deuteron) source is therefore the acid used in work-up. Conversion of the deuterated or tritiated saturated alcohols from GA_3 and GA_7 methyl ester to [^{2}H] and [^{3}H] GA_1, GA_5 and GA_{20} methyl esters and to [^{2}H] and [^{3}H]GA_4 and GA_9 methyl esters respectively, are shown in Fig. 8. The alkaline hydrolysis of these methyl esters to the free GAs is straightforward even for the 3-hydroxy GAs in which base-catalysed epimerisation occurs.

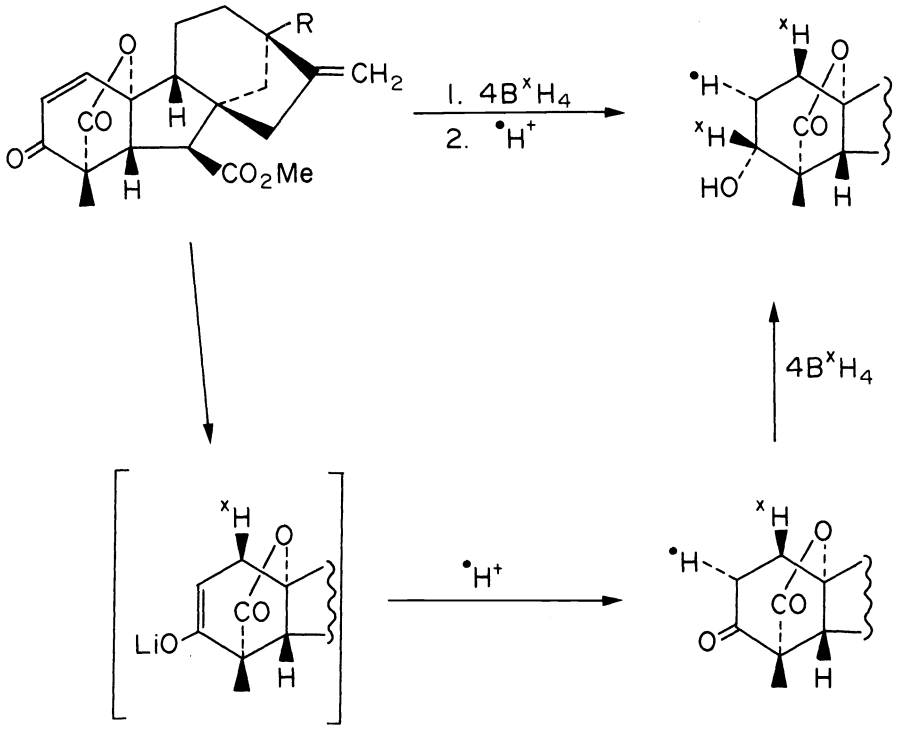


Fig. 7 Hydride reduction of 3-keto GA_3 and 3-keto GA_7 methyl esters

This epimerisation occurs without loss of the 3-deuterium or -tritium label eliminating 3-keto intermediate but supporting the previously suggested retro-aldol mechanism. The stereochemistry of the 3-label in the reduction of the enone (Fig. 7) follows from the

characterisation of the products as 3-epi GA_1 methyl ester (from GA_3 methyl ester) and 3-epi GA_4 methyl ester (from GA_7 methyl ester). The 1β,2α-[$^2H_2$]stereochemistry was determined by the conversion of [1,3-$^2H_2$]GA_4 into [1,3-$^2H_2$]GA_3, and of [1,2-$^2H_2$]GA_4 into [1-$^2H_2$]GA_3 by cultures of G. fujikuroi mutant B1-41a, Evans et al. (16) having previously shown that the 1,2-double bond in GA_3 was formed by loss of the 1α- and 2α-hydrogen atoms.

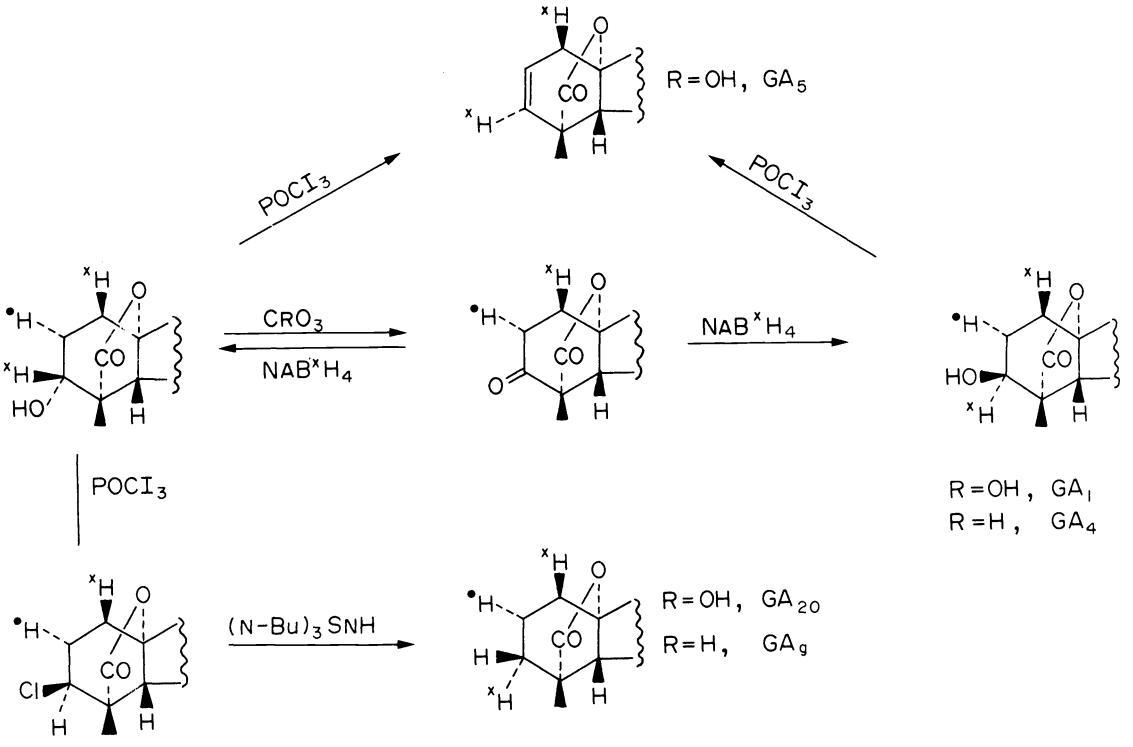


Fig. 8 Synthesis of [$^{x\cdot}$H]GAs (x and · = ^1H, ^2H, or ^3H).

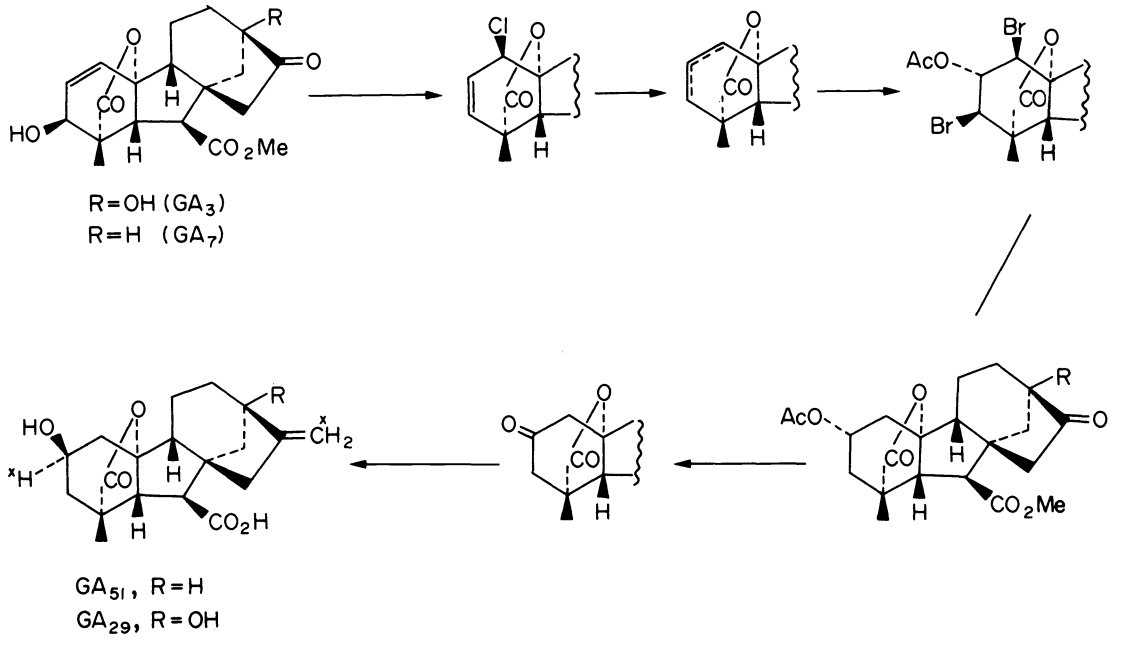


Fig. 9 Synthesis of [2-^{x}H]GA_{29} from GA_3 and [2-^{x}H]GA_{51} from GA_7.

A synthesis of $[2\alpha\text{-}^2H_1]GA_{29}$ and $[2\alpha\text{-}^2H_1]GA_{51}$ from GA_3 and GA_7 respectively is shown in Fig. 9.

These $[^2H]$GAs can be used, in conjunction with mass spectrometry, to solve many types of metabolic problems. For example, the $[^2H]$-metabolites can be identified in the presence of the same native compound (Ref. 11). The $[^2H]$GAs can be used as internal standards to quantify the levels of the native GAs. Combining these two applications, $[^2H]$GAs can be used in time-course studies. These applications are illustrated by two examples from our recent work on GA-metabolism in developing seed of P. sativum cv. Progress No. 9.

Firstly, in our earlier time-course studies (Ref. 7) the levels of the native GAs were determined by selected ion current monitoring in methylated and trimethylsilylated methanolic extracts. Recently Browning and Saunders (17) have reported that Triton X-100 is 1000 times more efficient than methanol in extracting GA_9 from chloroplast preparations from leaves of Triticum aestivum cv. Kolibri. In order to check the validity of our previous time-course data, we have compared the efficiency of Triton X-100 and methanol extraction of our pea seed. Known quantities of $[1\beta,3\alpha\text{-}^2H_2]GA_{20}$ and $[2\alpha\text{-}^2H_1]GA_{29}$ were added to methanolic and Triton X-100 extracts of seed at four developmental stages. After purification the $[^2H]:[^1H]$ ratios were determined by g.l.c.-mass spectrometry from the M^+ cluster of the MeTMSi derivatives of the extracts. From these ratios the total amounts of extractable GA_{20} and GA_{29} were calculated. Preliminary results (Table 1) clearly show that for pea seed, there is no difference in the amounts of GA_{20} and GA_{29} extracted by methanol and by Triton X-100 (unpublished results).

TABLE 1. Comparison of the amounts of GA_{20} and GA_{29} extracted from immature pea seeds by aqueous methanol and Triton X-100 (μg/seed).

Seed Age (days)	GA_{20}		GA_{29}	
	MeOH	Triton	MeOH	Triton
21	3.49	3.14	Not determined	
27	0.27	0.48	10.29	9.91
34	0.1	0.1	5.01	4.43

$[^2H]GA_{20}$ and $[^2H]GA_{29}$ used as internal standards.

Secondly, a known amount of $[1\beta,2\alpha\text{-}^2H_2]GA_{20}$ (admixed with sufficient $[^3H]GA_{20}$ to follow the subsequent fractionation steps) was injected into seed developing in intact pods on the plant at a stage immediately before the level of the native GA_{20} reached a maximum. A sample of the seed were immediately harvested and further samples were taken at 3 or 4 day intervals until the seed were fully grown. Each sample of seed was extracted with methanol and the $[^2H]$-content of the recovered GA_{20} and the recovered GA_{29} were determined as described in the preceding paragraph. These ratios (Table 2) show that there was a net synthesis of native GA_{20} from the time of feeding for about 2 days after the feed. There after the ratio of $[^2H]GA_{29}$:GA_{29} remained approximately constant, indicating that the applied $[^2H]GA_{20}$ is reaching the pool of the native GA_{20} (unpublished results).

TABLE 2. $[^2H][^3H]GA_{20}$ time-course feed to immature pea seeds: $[^2H]$data.

	Percentage of non-, mono-, and di-deuterated species in recovered GA*		
	$[^2H]_0$	$[^2H]_1$	$[^2H]_2$
GA_{20} substrate	11.96	10.57	77.47
GA_{20} day 0	39.14	6.75	54.11
GA_{20} day 2	57.67	5.18	37.15
GA_{20} day 5	75.77	3.44	20.80
GA_{20} day 9		None recovered	
GA_{20} day 13		None recovered	
GA_{29} day 0		None recovered	
GA_{29} day 2	51.36	4.95	43.68
GA_{29} day 5	72.10	3.35	24.55
GA_{29} day 9	79.24	1.99	18.77
GA_{29} day 13	78.24	2.29	19.48

*As calculated from the molecular ion cluster.

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