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The Identification of Gibberellins in Immature Seeds of *Vicia faba*, and Some Chemotaxonomic Considerations

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Abstract. GA_{17}, GA_{19}, GA_{20}, GA_{29}, GA_{44} and 13-hydroxy-GA_{12}, now named GA_{53}, were identified by GC-MS in immature seeds of *Vicia faba* (broad bean). Also identified were a GA catabolite, two polyhydroxykauranoic acids, and abscisic, phaseic and dihydrophaseic acids. The GAs of *Vicia* are hydroxylated at C-13, in common with those of other legumes. However the GAs of *Vicia* are not hydroxylated at C-3, nor do they appear to be readily conjugated. In these respects *Vicia* resembles *Pisum*, another member of the tribe *Viciae*. *Vicia* differs from *Phaseolus* and *Vigna*, of the tribe *Phaseoleae*, in both these respects.

Key words: Gas chromatography-mass spectrometry – Gibberellin – Hydroxylation – *Leguminosae* – Seeds (gibberellins) – *Vicia*.

Introduction

Of the 52 GAs known to date 42 have been identified in higher plants (Bearder, in press). These GAs differ from one another in the degree and position of oxidation within the molecule. A sufficiently wide variety of plants have been studied to conclude that there is a similarity in the position of oxidation of GAs identified in particular angiosperm families (Takahashi, 1974; Bearder and Sponsel, 1977; Graebe and Ropers, 1978). Thus whilst 13-hydroxylated GAs are predominant in members of the *Leguminosae*, they are absent in all members of the *Cucurbitaceae* studied to date (Bearder, in press). These correlations have been extended by the following identifications of free and conjugated GAs in *Vicia faba* (broad bean) in the family *Leguminosae*, tribe *Viciae*.

Abbreviations: ABA = abscisic acid; DPA = dihydrophaseic acid; GA_n = gibberellin A_n; GC = gas chromatography; GC-MS = gas chromatography mass spectrometry; KA = kauranoic acid; PA = phaseic acid; TLC = thin layer chromatography

Materials and Methods

Plant Material

Pods of *Vicia faba* were purchased from commercial sources and were of unknown cultivar. Seeds which were sorted into "small" and "intermediate" categories were obtained from pods imported from Spain in March. "Large" seeds were obtained from locally grown plant material in June. Mean seed weights were "small" = 0.89 g/seed, "intermediate"-sized = 1.40 g/seed, and "large" = 2.50 g/seed. Seeds were deep frozen at −15° C until use.

Extraction and Purification

Seeds (130 g fresh weight of "small", 100 g fresh weight of "intermediate"-sized, and 73 g fresh weight of "large") were ground in 80% aqueous methanol and were extracted three times with the same solvent. The combined filtrates were pooled, reduced in volume to the aqueous phase and were chromatographed on Dowex 1X1-100 (formate) anion exchange resin. Gibberellins were eluted with ethanol:formic acid (4:1) as described by Browning and Saunders (1977). Column eluates were reduced to aqueous and were partitioned against ethyl acetate at pH 8, and against ethyl acetate and then n-butanol at pH 3. The initial acidic ethyl acetate extract was reduced to dryness in vacuo, weighed and redissolved in a small volume (ca. 5 ml) of 80% aqueous methanol before partitioning three times against equal volumes of petroleum ether (b.p. 60–80). The methanolic phase was reduced to dryness and the residue, redissolved in pH 8 buffer (ca. 5 ml), was partitioned three times against benzene. Acidification of the aqueous phase to pH 3 and further partitioning three times against ethyl acetate afforded the final acidic ethyl acetate fraction.

Thin Layer Chromatography

Extracts were strip-loaded onto 20 × 20 cm plates of prewashed silica gel HF (Merck), 0.4 mm thickness, and developed with ethyl acetate: chloroform: acetic acid (15:5:1). Plates were divided into four equal bands (called zones 1–4 numbering consecutively from the origin) and material was eluted from the silica gel of each

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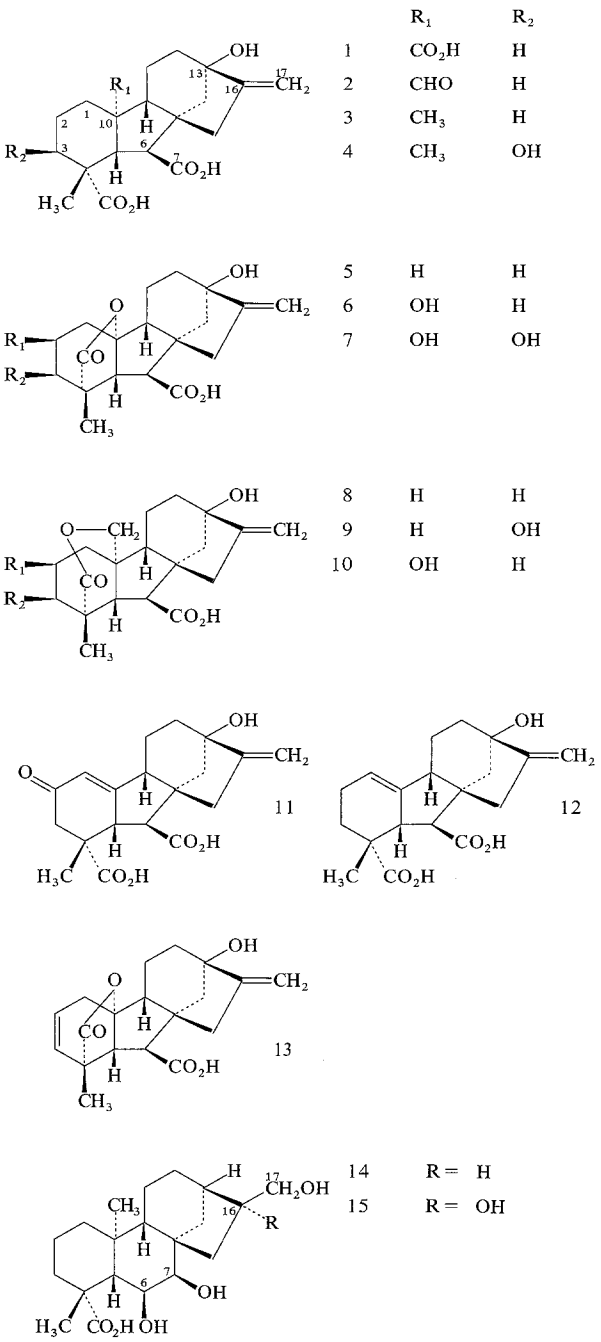

band with water-saturated ethyl acetate. All traces of silica were removed by repeated centrifugation.

Enzyme Hydrolysis

Hydrolysis of butanol extracts, and extraction of hydrolysates were conducted as described by Frydman and MacMillan (1975). The enzyme was removed from its Kieselguhr support prior to use. Acidic ethyl acetate fractions of the hydrolysates were derivatized and gas chromatographed directly in the case of "small" and "intermediate"-sized seed extracts. For the "large" seed extract the corresponding fraction was chromatographed on silica gel plates and four equal zones were eluted, derivatized and gas chromatographed.

Gas Chromatography and Gas Chromatography-Mass Spectrometry

Extracts were derivatized and gas chromatographed as described in Frydman and MacMillan (1975). Chromatography was performed on 2% SE-33 and sometimes on 2% QF-1 columns, at 190° for the first 5 min then increasing at 3° min^{-1}. GC-MS was performed as described previously (Frydman et al., 1974).

Results

Free GAs and Other Acids

Seeds of *Vicia faba* of three sizes (as estimated by fresh weight and designated "small", "intermediate" and "large") were extracted as described in the Materials and Methods section. GC and GC-MS of derivatized aliquots of initial acidic ethyl acetate fractions showed large quantities of fatty acids and other lipids. These precluded the identification of GAs. 75% Reduction in dry weight of these fractions was achieved by further partitioning with petroleum ether and benzene as described in the Materials and Methods section. The final acidic ethyl acetate fractions were chromatographed on silica gel plates. Material from each of the TLC zones 1–3 (Rf 0–0.75) from each extract was derivatized and gas chromatographed. GC-MS identifications are shown in Table 1.

Several GAs were conclusively identified in the final acidic ethyl acetate fraction of "small" seeds, namely the MeTMS derivatives of GA_{17}(1), GA_{19}(2), GA_{20}(5), GA_{29}(6), and GA_{44}(8). As in *Pisum sativum* (garden pea) (Frydman et al., 1974) GA_{20} and GA_{29} were the major GAs in this extract. The GA catabolite (11), recently identified in pea (Sponsel and MacMillan, 1978) was also present as the MeTMS derivative. Trace amounts of 13-hydroxy GA_{12}(3), known to be a metabolite in cell-free systems from pea (Ropers et al., 1978) were present in zone 3. Since this GA has been fully characterized (Bearder et al., 1975) and is now known to be an endogenous compound it is allocated the name GA_{53} (MacMillan and Takahashi, 1968). GA_{53} is also known to occur in apple (Hoad, 1978) and maize (Hedden et al., unpublished). Zone 3 also contained a compound tentatively assigned the structure (12) and previously shown to occur in pea (Sponsel and MacMillan, 1978).

Several other tentative identifications of minor components of the "small" seed extract were made on the basis of their mass spectra. Zone 2 contained

Table 1. Identification of GAs, Kauranoids, ABA and Related Compounds in *Vicia faba* (unknown cultivar)

Seed size	Fraction	GAs	Kauranoids	ABA and related compounds
Small	final acidic ethyl acetate	GA_{17} GA_{19} GA_{20} GA_{29} GA_{44} GA_{53} compound (11)	6β,7β,17-tri-OHKA 6β,7β,16,17-tetra-OHKA	ct and tt ABA ct and tt DPA ct PA
	hydrolysed acidic butanol	GA_{29}	6β,7β,16,17-tetra-OHKA	ct and tt DPA
Intermediate	final acidic ethyl acetate	GA_{20} GA_{29} compound (11)	6β,7β,17-tri-OHKA 6β,7β,16,17-tetra-OHKA	ct ABA ct and tt DPA
	hydrolysed acidic butanol	GA_{17} GA_{29}		ct and tt DPA
Large	final acidic ethyl acetate	GA_{20} GA_{29} compound (11)	6β,7β,17-tri-OHKA 6β,7β,16,17-tetra-OHKA	ct and tt DPA
	hydrolysed acidic butanol	GA_{17} GA_{20} GA_{29} GA_{53} compound (11)	6β,7β,17-tri-OHKA 6β,7β,16,17-tetra-OHKA	ct ABA ct and tt DPA

a compound giving a spectrum which was reminiscent of that of MeTMS GA_{38}. This spectrum is also similar to that of a compound in pea previously identified as GA_{38}(9) (Frydman et al., 1974). It is possible that this compound of pea was misidentified and has instead the isomeric structure (10). Until this hitherto unknown compound has been synthesised for comparison with that in pea and broad bean, the identification of GA_{38} in pea is withdrawn.

Zone 2 of the "small" seed extract contained a compound with a mass spectrum similar in many respects to that of the MeTMS derivative of GA_{18}(4), of which it may be an isomer. In this zone there was also an indication of the presence of trace amounts of a trihydroxy GA_9 derivative. It did not appear to be GA_8(7).

In zone 3 the presence of an unusually large signal at m/e 416 in the spectrum of MeTMS GA_{20} was an indication that a trace of MeTMS GA_5 might be present. GA_5(13) and GA_{20}(5) are not separable as the MeTMS derivatives with the GC conditions used here. Horgan and Heald (unpublished, quoted in Bearder and Sponsel, 1977) have identified GA_5 in addition to GA_{20} and GA_{44} in *V. faba*.

The final acidic ethyl acetate fraction of "small" seeds also contained two polyhydroxykauranoic acids (14) and (15), and ABA and related compounds as detailed in Table 1.

Extracts of "intermediate"-sized and "large" seeds contained fewer GAs than "small" seeds. Traces of GA_{20}(5) and a little GA_{29}(6) were present in both extracts. The GA catabolite (11), the polyhydroxykauranoic acids (14) and (15) and dihydrophaseic acid were also present (Table 1). "Intermediate"-sized seeds contained ABA and "large" seeds the putative (12) too.

Conjugated GAs and Related Compounds

The acidic butanol fractions of extracts of the three seed sizes were subjected to enzyme hydrolysis as described in the Materials and Methods section. GC-MS identifications are shown in Table 1.

In contrast to the free acidic GAs discussed above, the "large" seed extracts were the richer source of GAs after hydrolysis. These are presumably present initially as conjugates. "Large" seed hydrolysates contained traces of GA_{20}(5), GA_{53}(3), and small quantities of GA_{17}(1), GA_{29}(6) and the catabolite (11). Traces of an isomer of GA_8 and the GA_{20} derivative (12) were tentatively identified. Traces of ABA, large quantities of DPA and some of the polyhydroxykauranoic acids (14) and (15) were also present.

The hydrolysates of "small" and "intermediate"-sized seeds contained traces of GA_{29}(6), the putative

Table 2. A Comparison of the GAs of Several Species of *Leguminosae*, in Relation to 3-hydroxylation

Tribe	Species	C19-GAs		C20-GAs		Reference
		3-H	3-OH	3-H	3-OH	
Viciae	*Pisum sativum*	GA_9,GA_{20}, GA_{29},GA_{51}, compound (11)	–	GA_{17},GA_{44}		See Sponsel and MacMillan (1977)
	Vicia faba	GA_5,GA_{20}, GA_{29}, compound (11)	–	GA_{17},GA_{19}, GA_{44},GA_{53}	–	Present paper and Horgan and Heald (unpublished)
Phaseoleae	*Phaseolus vulgaris*	GA_5,GA_{20}, GA_{29}	GA_1,GA_4, GA_6,GA_8	GA_{44}	GA_{37},GA_{38}	See Yamane et al. (1977)
	Phaseolus coccineus	GA_5,GA_{20}	GA_1,GA_3,GA_4, GA_6,GA_8,GA_{34}, 3-OH-compound (11)	GA_{17},GA_{19}, GA_{44}	GA_{28},GA_{38}	Durley et al. (1971) and Sponsel and Albone (unpublished)
	Vigna unguiculata	GA_{20}	GA_4,GA_6,GA_8, 3-OH-compound (11)	GA_{17}		Adesomoju (1977) and Gaskin (unpublished)

(12) and a lot of DPA. In addition, that of small seeds contained the trihydroxykauranoic acid and that of "intermediate"-sized seeds contained a trace of GA_{17} (1) (Table 1).

Discussion

Of the six GAs identified in immature seeds of *Vicia faba* five of them, namely GA_{17}(1), GA_{19}(2), GA_{20}(5), GA_{29}(6) and GA_{44}(8) are GAs commonly found in legumes (Table 2). This is a reinforcement of the earlier observations by Takahashi (1974), Bearder and Sponsel (1977) and Graebe and Ropers (1978) on the structural similarity of GAs identified in related species. Thus most GAs identified in members of the family *Leguminosae* are 13-hydroxylated. In contrast 13-hydroxylated GAs are apparently absent in all members of the *Cucurbitaceae* studied to date. The identification of GA_{53} (13-hydroxy GA_{12}) in other legumes can be anticipated.

Within the *Leguminosae*, comparison of GA type may be possible at a further taxonomic level. Firstly, immature seeds of *Phaseolus coccineus* (runner bean), *Ph. vulgaris* (French bean), and *Vigna unguiculata* (cow pea), in the tribe Phaseoleae (Heywood, 1971) contain many GAs which are also 3-hydroxylated (Durley et al., 1971; Hiraga et al., 1974a and references therein; and Adesomoju, 1977). In contrast immature seeds of *Pisum sativum* (Sponsel and MacMillan, 1977), and *Vicia faba*, which belong to very closely related genera in the tribe *Viciae* (Heywood, 1971; Marx, 1977) contain non-3-hydroxylated GAs. Table 2 contrasts the GAs of the above species in relation to 3-hydroxylation.

Secondly, mature seeds of *Phaseolus* species are rich sources of conjugated GAs (Gaskin and MacMillan, 1975; Hiraga et al., 1974b). Conversely those of *P. sativum* (Sponsel and MacMillan, 1977) and *V. faba* appear to contain scarcely any GA conjugates, in so far as very low levels of GAs are observed in enzyme hydrolysates of appropriate fractions. A GA catabolite (11) recently identified as a major component in *P. sativum* seeds (Sponsel and MacMillan, in press) is shown here to be endogenous to *V. faba* seeds too. This suggests that in *Vicia* and *Pisum* an alternative mechanism to conjugation exists for disposing of GAs. Re-examination of *Vigna unguiculata* extracts (Gaskin, unpublished) and further extraction of *Phaseolus coccineus* seeds and pods (Sponsel and Albone, unpublished) have indicated the presence of traces of a 3-hydroxylated derivative of compound (11) (Gaskin, Kirkwood and MacMillan, unpublished). Metabolic studies are required to determine if in members of the *Viciae* the catabolic pathway through compound (11) is predominant, and if in members of the *Phaseoleae* conjugation is the major route for GA disposal.

The vast quantity of DPA present in butanol hydrolysates of *Vicia* seeds, and the presence of several unidentified compounds which from their mass spectra appear to be related to ABA, indicate that *Vicia* seeds would be ideal material for a study of ABA metabolism.

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