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Gibberellins in seedlings and flowering trees of *Prunus avium* L.

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Abstract

Extracts of acids from mature seeds, germinating seeds, first, second and third year seedlings as well as mature, flowering trees of sweet cherry (*Prunus avium* L. cv. Stella) were analysed by gas chromatography–mass spectrometry. The presence of the known gibberellins (GAs) GA_1 (**1**), GA_3 (**4**), GA_5 (**7**), GA_8 (**11**), GA_{19} (**14**), GA_{20} (**12**), GA_{29} (**13**), GA_{32} (**5**), GA_{85} (**2**), GA_{86} (**3**) and GA_{87} (**6**) was confirmed by comparison of their mass spectra and Kovats retention indices with those of standards or literature values. In addition, 16α,17-dihydrodihydroxy GA_{25} (**16**) was identified and its stereochemistry confirmed by rational synthesis. The 12α,13-dihydroxy GAs, GA_{32} (**5**), GA_{85} (**2**), GA_{86} (**3**) and GA_{87} (**6**), were detected in mature seeds, germinating seeds and young seedlings, but not in flowering plants. The 13-hydroxy GAs, GA_1 (**1**) and GA_3 (**4**), were present in germinating seeds and, in addition to these, GA_5 (**7**), GA_8 (**11**), GA_{19} (**14**), GA_{20} (**12**) and GA_{29} (**13**) were detected in seedlings and mature flowering plants. In germinating seeds and seedlings (while the plants were growing actively), concentrations of the 12α,13-dihydroxy GAs, measured by bioassay, declined and those of the 13-hydroxy GAs increased. The results are discussed with reference to the known and predicted effects of the GAs on the vegetative growth and flowering of *P. avium* plants.

Keywords: *Prunus avium*; *Rosaceae*; Sweet cherry; Gibberellins; Identification; Juvenility; Phase-change

1. Introduction

Generally, the production of flowers by a woody plant is the most obvious sign that it has passed through the juvenile and into the mature phase of development. Most wild *Prunus avium* (Mazzard) seedlings first begin initiating floral buds during their fourth growing season after germination (i.e. as three-year-old plants) and flower the following spring, although some initiate flower buds and flower one year earlier (Schmidt, 1976). A 'physiological' distancing of the shoot meristems from root produced floral inhibitors has been invoked to explain this dependence of the ability to flower on plant size (Hackett, 1985). This view is supported by experiments where grafting to mature flowering trees stimulated juvenile cherry scions to initiate floral buds during their third growing season after germination and, conversely, initiation of floral buds was inhibited completely in mature scions when they were grafted to juvenile seedling rootstocks (Olivera and Browning, 1993a). These observations led Olivera & Browning (1993a) to suggest that phase change was reversible and was controlled by the transport of an unknown substance or substances from the roots towards the shoot apex that inhibited flowering and it was noted that the substance was graft transmissible.

Possible candidates for root-produced floral inhibitors are the gibberellins (GAs) (Wareing & Frydman, 1976). Some GAs, when applied exogenously, inhibit flowering in many woody angiosperms (Ross, Pharis & Binder, 1983; Zimmerman, Hackett & Pharis, 1985; Metzger, 1987), including *P. avium* (Hull & Lewis, 1959; Bradley & Crane, 1960; Rebeiz & Crane, 1961;

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Table 1
Lettuce hypocotyl growth in response to extracts of *P. avium* tissues (100 seeds or seedlings) after fractionation by HPLC[a]

HPLC fraction	Mature seed	Germinating seed	Seedlings 5 weeks	Seedlings 10 weeks	Seedling 15 weeks	Seedling 25 weeks
13–14	+ + + + +	+ + + + + +	+ + + +	+ + +	+ +	+
16–18	+ + + +	+ + + + +	+ + +	+	–	–
22–24	–	+ +	+ + +	+ + +	+ +	–

[a] + + + + + + = 15–20 mm = 3.0 μg GA_3; + + + + + = 12–15 mm = 1.0 μg GA_3; + + + + = 9–12 mm = 0.3 μg GA_3; + + + = 6–9 mm = 0.1 μg GA_3; + + = 4–6 mm = 0.03 μg GA_3; + = 2–4 mm = 0.01 μg GA_3; – = 0–2 mm = water control.

Proebsting & Mills, 1974; Goldwin & Webster, 1978; Facteau, Rowe & Chestnut, 1989; Olivera & Browning, 1993b). The effects of exogenous GAs on the flowering of woody plants and their ability to induce the development of juvenile characters in some mature woody plants has prompted the suggestion that changes in the GA supply, for example from the roots to the shoots, are involved in the mechanism of phase change (Zimmerman et al., 1985). Also, the endogenous GA status, measured by bioassay, of juvenile versus mature materials has been found to differ in several woody species (Zimmerman et al., 1985) and, in the one reported study using gas chromatography–mass spectrometry (GC–MS), shoots from young plants of Sika spruce (*Picea sitchensis*) contained predominantly GA_4 (**9**) and GA_9 (**10**) while physiologically mature, flowering trees contained mainly GA_1 (**1**) and GA_3 (**4**) (Moritz, Philipson & Oden, 1989a).

To date, immature fruitlets of sweet cherry (*P. avium*) (Blake, Browning, Chu & Mander, 1993) and immature seeds of sour cherry (*P. cerasus*) (Nakayama et al., 1996) are the only tissues in which GA_{32} (**5**) and GA_{87} (**6**) have been identified unequivocally by GC–MS and Kovats retention indices (KRI). GA_1 (**1**), GA_3 (**4**), GA_5 (**7**), GA_8 (**11**), GA_{19} (**14**), GA_{20} (**12**), GA_{29} (**13**), GA_{85} (**2**) and GA_{86} (**3**) are also present in immature fruitlets of sweet cherry (Blake & Browning, 1994) and, in addition to these, GA_{95} (**17**) was identified in immature seeds of sour cherry (Nakayama et al., 1996). Other studies have identified GA_5 (**7**), GA_{32} (**5**) and GA_{32} acetonide in immature seeds of peach (*P. persica* L.) (Yamaguchi, Yokota, Murofushi, Takahashi & Ogawa, 1975) and GA_1 (**1**), GA_5 (**7**), GA_{29} (**13**) and GA_{32} (**5**) were identified tentatively by GC–MS–Selected Ion Monitoring (SIM) in immature seeds of apricot (*P. armeniaca* L.) (Bottini, Bottini, Koshioka, Pharis & Coombe, 1985). The GAs in vegetative tissues of *Prunus* species have been identified tentatively as GA_1 (**1**), GA_3 (**4**) and GA_8 (**11**) by GC–SIM in flower buds of peach (Luna et al., 1990) and GA_1 (**1**) and GA_3 (**4**) were quantified in the xylem sap of peach trees using HPLC and radioimmunoassay (Cutting & Lyne, 1993). Studies of GA metabolism using cell suspension cultures of peach leaves, with GC–MS–SIM, showed that $[^2H_2]$-GA_5 was converted into free deuteriated GA_1 (**1**), GA_3 (**4**), GA_6 (**18**), GA_8 (**11**) and GA_{22} (**8**) in addition to various conjugates (Koshioka, Pharis, Matsuta & Mander, 1988).

The aims of the experiments reported here were to characterise, by full-scan GC–MS, the GAs in ripe seeds, germinating seeds, young seedlings and apices of juvenile and physiologically mature plants. Thus, identifying the qualitative differences in the GAs of juvenile and mature tissues and thereby providing targets

Table 2
Lettuce hypocotyl growth in response to extracts (100 shoot tips) of *P. avium* of different ages after fractionation by HPLC[a]

HPLC fraction	Weeks from germination[b] or leaf emergence[c]															
	1st year[b]				2nd year[c]				3rd year[c]				Mature[c]			
	5	10	15	25	5	10	15	25	5	10	15	25	5	10	15	25
13–14	+ + + +	+ + +	+ +	+	–	–	–	–	–	–	–	–	–	–	–	–
16–18	+ + +	+ +	+	–	–	–	–	–	–	–	–	–	–	–	–	–
22–24	+ +	+ + +	+ +	+	+ + + +	+ + + +	+ +	–	+ + +	+ + + +	+ +	–	+ +	+ + +	+	–

[a] + + + + + + = 15–20 mm = 3.0 μg GA_3; + + + + + = 12–15 mm = 1.0 μg GA_3; + + + + = 9–12 mm = 0.3 μg GA_3; + + + = 6–9 mm = 0.1 μg GA_3; + + = 4–6 mm = 0.03 μg GA_3; + = 2–4 mm = 0.01 μg GA_3; – = 0–2 mm = water control.
[b] From germination.
[c] From leaf emergence.

Table 3
Kovats retention indices (KRI) and relative intensities of characteristic ions for MeTMSi derivatives of Gibberellins from *P. avium* tissues and standard compounds

Identified GA	Kovats retention index		Diagnostic ions (m/z) with abundance in reference and sample								HPLC fraction	Source[a]
32		Ion	680(M^+)	665	590	546	500	456	397	339		
	2992	Sample	9	18	100	20	26	25	27	65	13–14	MS, GS, S
86		Ion	682(M^+)	667	592	548	533	302	463	339		
	2996	Sample	10	10	100	7	13	35	5	15	13–14	MS, GS, S
87		Ion	592(M^+)	548	502	489	355	238	193	147		
	2894	Standard	15	4	6	25	16	22	39	100		
	2895	Sample	16	5	10	23	14	18	29	100	16–17	MS, GS, S
85		Ion	594(M^+)	579	550	504	491	445	375	348		
	2858	Sample	31	7	7	42	100	11	35	70	17–18	MS, GS, S
8		Ion	594(M^+)	579	535	504	448	379	375	238		
	2848	Sample	100	7	5	5	26	22	17	35	17–18	S, M
29		Ion	506(M^+)	491	447	375	303	281	235	207		
	2749	Sample	100	10	10	25	20	9	18	41	18–19	S, M
3		Ion	504(M^+)	489	475	445	387	370	355	208		
	2763	Standard	100	10	17	22	12	24	19	50		
	2763	Sample	100	11	19	18	18	22	25	45	22–23	GS, S, M
1		Ion	506(M^+)	491	448	377	376	313	235	207		
	2744	Standard	100	14	25	30	30	25	24	55		
	2744	Sample	100	8	20	27	25	20	20	65	23–24	GS, S, M
20		Ion	418(M^+)	403	389	375	359	301	235	207		
	2580	Standard	100	14	10	70	25	25	16	37		
	2580	Sample	100	12	11	67	24	27	14	40	30–31	GS, S, M
5		Ion	416(M^+)	401	385	372	257	343	299	207		
	2576	Standard	100	25	5	5	15	14	56	51		
	2576	Sample	100	24	8	10	31	19	47	48	31–32	GS, S, M
19		Ion	462(M^+)	434	402	375	374	345	285	208		
	2682	Standard	4	100	52	78	96	41	38	47		
	2681	Sample	4	100	45	70	89	40	27	46	34–36	GS, S, M
16α,17$(OH)_2$ GA_{25}		Ion	582(M^+)	567	479	447	419	387	359	147		
	2924	Standard	2	8	100	10	89	68	52	45		
	2923	Sample	2	7	100	7	75	55	44	57	26–27	MS, GS

[a] MS = mature seeds; GS = germinating seeds; S = seedlings (10 weeks old); M = mature, flowering plants.

for a quantitative study of GAs and their role in the control of phase change in *P. avium* trees.

2. Results and discussion

GAs were extracted and purified by the method reported for the isolation of GA_{87} (**6**) (Blake et al., 1993), with the modifications described for purification of GAs from fruitlets of *P. avium* (Blake & Browning, 1994). HPLC fractions that showed GA-like activity in the lettuce hypocotyl bioassay (see Tables 1 and 2) were derivatised and analysed by GC–MS. Extracts prepared from vegetative tissues required an additional TLC step before samples could be analysed by GC–MS, as many HPLC fractions yielded milligram quantities of white crystalline solid after evaporation to dryness. Some of these fractions showed GA-like activity in the bioassays but, when derivatised and analysed by GC–MS, the spectra contained ions of high intensity characteristic of carbohydrate TMSI derivatives (*m*/*z* 147, 191, 204, 217 and 361) (Gaskin & MacMillan, 1991), while spectra of GA MeTMSI derivatives were not detectable. Such extracts were purified further by TLC and although this resulted in losses of GAs (ca. 40% loss of the added [^{3}H]-GA_1), the reduction in sample dry weight and the consequent increase in GA concentration were sufficient to allow unequivocal identification by comparison of mass spectra and KRI of MeTMSI derivatives obtained, with those of standards or literature values (Gaskin & MacMillan, 1991) (Table 3).

The 12α,13-dihydroxy GAs, GA_{32} (**5**) and GA_{86} (**3**), were found in fractions 13–14, GA_{85} (**2**) and GA_{87} (**6**) in fractions 16–18, while GA_1 (**1**) and GA_3 (**4**) (the biologically active products of the early 13-hydroxylation pathway) were present in fractions 22–24. HPLC fractions in which no GA-like activity was detected by the bioassays were also derivatised and analysed by GC–MS. When the KRIs and full-scan spectra of MeTMSI derivatives were compared with literature values (Gaskin & MacMillan, 1991) or values for standard compounds obtained under the same GC–MS conditions, GA_5 (**7**), GA_8 (**11**), GA_{19} (**14**), GA_{20} (**12**) and GA_{29} (**13**) were identified (Table 3). These results indicate that the early 13-hydroxylation pathway of GA biosynthesis is active in vegetative tissues of sweet cherry. But, as in immature fruitlets (Blake & Browning, 1994), the earlier members of the pathway (GA_{12} (**19**), GA_{53} (**15**) and GA_{44} (**20**) were not detectable either by full-scan or SIM mass spectrometry. In addition to the 12α,13-dihydroxy and 13-hydroxy GAs present in the extracts of mature and germinating seeds, examination of the total ion chromatogram, obtained from the MeTMSI derivatives of fractions 26 and 27, revealed the presence of a compound at KRI 2923, exhibiting a weak molecular ion *m*/*z* 582, and a base peak *m*/*z* 479. The loss of *m*/*z* 103 indicated strongly that the parent molecule contained a 16,17-dihydrodiol group (Gaskin & MacMillan, 1991) and, by comparison of the full-scan spectrum and KRI data (Table 3) with literature values (Gaskin & MacMillan, 1991), the methyl ester TMSI derivative of 16α,17-dihydro-dihydroxy-GA_{25} (**16**) was identified.

It was not possible, however, to determine the stereochemistry at **C-16** from the mass spectrum and KRI. An authentic sample of the GA_{25} diol trimethyl ester was prepared by dihydroxylation of GA_{25} trimethyl ester (prepared from GA_{13} (**21**) by the method of Fraga, Gonzalez, Tellado, Duri and Hanson (1984) with osmium tetroxide, using 4-methylmorpholine N-oxide as a co-oxidant (Van Rheenan, Kelly & Cha, 1976). The 16α-stereochemistry was assigned to the product in the expectation that the reagent would approach from the less hindered exo-face of the D-ring; this was confirmed by consideration of the ^{13}C-NMR spectrum of the product. The resonance for **C-12** was observed at δ 21.9, compared with δ 31.5 in GA_{25} trimethylester, the strong upfield shift being a consequence of the γ-gauche effect exerted by the endo-hydroxymethyl group. The KRI and mass spectra of the product were identical to those of the endogenous compound (Table 3).

A comparison of the concentrations of dihydro-dihydroxy GA_{25} in extracts of equal numbers of seeds and seedlings was made by measuring peak areas in the total ion current chromatograms (data not shown). From these measurements, the concentrations of the dihydrodiol in the seeds decreased during stratification and the GA was not detectable in seedlings, 5 weeks after germination. It is possible that this GA has a role in dormancy and/or transition to germination of seeds. However, the 16α,17-dihydro-dihydroxy derivatives of GA_4, GA_7, GA_9 and GA_{12} were identified in developing apple seeds by Hedden et al. (1993) and they suggested that these compounds resulted from the non-specific oxidation of the 16,17-double bond and were prevalent for GAs and precursors of low polarity. A biological function has not yet been assigned to this group of GAs.

The lettuce hypocotyl bioassay was used to estimate the relative amounts of the 12α,13-dihydroxy GAs, GA_{32} (**5**), GA_{85} (**2**), GA_{86} (**3**) and GA_{87} (**6**), in HPLC fractions from mature seeds and seedlings. When deuteriated standards are available (from the GA synthesis program at the Australian National University), the amounts of these GAs in these tissues will be reassessed by GC–MS–SIM. The tetrahydroxy GAs GA_{32} (**5**) and GA_{86} (**3**) eluted in fractions 13 and 14, respectively but they were not resolved fully by the HPLC conditions used. The trihydroxy GAs also co-eluted, with GA_{87} (**6**) in fractions 16–17 and GA_{85} (**7**)

in fractions 17–18. Because of this, fractions 13–14 were combined for bioassay, as were fractions 16–18. Fractions 22–24 contained the dihydroxy GAs, GA_1 (**1**) and GA_3 (**4**), that are products of the 13-hydroxylation pathway. The results of bioassays were expressed as μg GA_3 equivalent for the combined fractions (Tables 1 and 2).

The strongest GA-like activity was associated with the 12α,13-dihydroxy GAs and occurred in the extracts of stratified, germinating seeds. However, only germinating seeds, with radicles 5–10 mm long, were selected for extraction and, as the germination rate was only ca. 50% this selection resulted in the exclusion of seeds with low viability. If concentrations of GAs were lower in seeds that were not viable, this might account for the weaker GA-like activity in samples of mature, but unstratified seeds. The 13-hydroxy GAs were not detected in extracts of mature seeds by bioassay (Table 1) or by GC–MS (Table 3) but, after stratification, the extracts of germinating seeds showed weak to moderate GA-like activity (Table 1) associated with the occurrence of GA_1 (**1**) and GA_3 (**4**) (Table 3). The GA-like activity increased while the seedlings were growing actively, but declined as growth terminated and winter dormancy approached (Tables 1 and 2).

Olivera and Browning (1993a) showed that floral initiation had occurred by the 11th July 1990 (15 weeks after full-bloom) in the sweet cherry cv. Stella growing in the UK and this coincides with the time when GA levels are decreasing (Table 2) as shoot growth is decreasing. Olivera and Browning (1993a) also demonstrated that GA_3 (**4**) treatments applied between the points of bud insertion in the distal area of individual fruiting spurs after full-bloom but before the initiation of floral buds prevented flowering in *P. avium*, while GA_1 (**1**) did not. Further work is needed to quantify GA_1 and GA_3 in seedlings and mature plants by GC–MS, during the time when flower initiation occurs and exogenous GAs can inhibit flower bud formation.

The presence of GA_{32} (**5**) and GA_{87} (**6**) in *P. avium* seeds and seedlings is of considerable interest because their chemical structures, with **C-1**,**2** unsaturation and a high degree of hydroxylation, were identified as those likely to have strong anti-florigenic activity and to promote seedling growth (Olivera & Browning, 1993b). Also, Olivera and Browning (1993a) noted that GA_3 treatments stimulated 44% of floral spurs to produce vegetative shoots. While applications of GA_{87} (**6**) to fruiting spurs of *P. avium* were as active as GA_3 (**4**) in inhibiting flowering, up to 80% of floral spurs treated with GA_{87} produced vegetative shoots (Blake, Chu and Mander unpublished results); it would be interesting to study this transition further. In contrast, Pharis, Evans, King and Mander (1987) and Evans, King, Chu, Mander and Pharis (1990) showed that, in *Lolium temulentum*, florigenic activity was increased by a **C-1**,**2** double bond (present in GA_{32} and GA_{87}), by hydroxylation at **C-3**, **C-12** and **C-13** (GA_{87}) and by **C-3**, **C-12**, **C-13** and **C-15** hydroxylation (GA_{32}). It has also been demonstrated that exogenous GA usually promotes flowering in conifers (Ross et al., 1983).

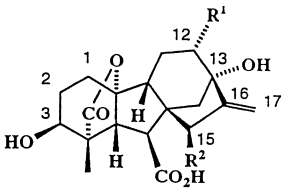
The bioassay results in this study suggested that most of the GA-like activity found in extracts of germinating seeds and young seedlings was attributable to the 12α,13-dihydroxy GAs, while in extracts of older seedlings and mature plants the activity was associated with the 13-hydroxy GAs. With the exception of the sample collected 25 weeks from leaf emergence or germination, our samples were taken during the period when exogenous gibberellins are known to affect initiation of flowers in *P. avium* (Olivera & Browning, 1993b). Generally, our results show that GA concentrations are high during spring and early summer while shoots are growing, as growth terminates GA levels decline and flower buds are initiated in florally competent spurs. While in a GC–MS study of GAs in sitka spruce (*Picea sitchensis*) (Moritz et al., 1989a) showed that GA_4 and GA_9 were the predominant GAs in juvenile non-flowering plants, while GA_1 and GA_3 were predominant in mature flowering trees. But, GA_1 and GA_3 were not detected and GA_4 and GA_9 (and particularly a GA_9 conjugate) were detected in clones of Sitka spruce that flowered well, while greater concentrations of GA_1 and GA_3 were detected in poorly-flowering clones (Moritz, Philpson & Oden, 1990). Suggesting that GA status alone is not responsible for phase-change in conifers.

While it is possible that the initial, rapid vegetative growth and lack of flowering in seedlings of *P. avium* are due partly to the presence of GA_{32} and GA_{87}. Our bioassay and GC–MS results showed that the influence of GA_{32} and GA_{87} declined rapidly during the first growing season and flowers are not initiated for a further 2–3 years. However, we were sampling shoot apices, while the first flowers a young cherry tree normally initiates are in axial buds on one-year-old shoots. It is possible that GA_{32} and GA_{87} are still present in or available to these axial buds although they are no longer detectable in shoot apices. Alternatively, GA_{32} and GA_{87} are active in concentrations that are not readily detectable by bioassay and GC–MS using deuteriated internal standards is required to test this hypothesis. However, the possibility that other hormonal mechanisms exist for controlling transition to the mature flowering state cannot be discounted. The source of the 12α,13-dihydroxy GAs is not clear, but they are present in the seeds of ripe fruits and their concentrations, measured by bioassay (Table 1), increased slightly through stratification. Following germination, the amounts of these GAs declined through the first growing season (Table

1). By the time winter dormancy was established, they were not detected by bioassay (Table 2). It is possible that they are biosynthesised from the 13-hydroxy GAs as was suggested previously (Blake et al., 1993), and as seedlings mature their GA metabolism is affected, as was shown for sitka spruce (Moritz, Philipson & Oden, 1989b). Alternatively, the 12α,13-dihydroxy GAs are products of an uncharacterised biosynthetic pathway in developing fruits and possibly in seedlings of *Prunus* spp.

The work presented here suggests that the 12α,13-dihydroxy GAs may have a role in maintaining the juvenile, non-flowering phase in sweet cherry seedlings and this needs further investigation. Although the source of the 12α,13-dihydroxy GAs is uncertain, the evidence available from this report and previous work (Blake et al., 1993; Blake & Browning, 1994; Nakayama et al., 1996) suggests that these GAs are biosynthesised in the developing fruit/seed and that they may be 'carried over' into the seedling. This hypothesis is supported by indirect evidence from the literature. For example, when GA biosynthesis inhibitors such as paclobutrazol are applied to developing *P. avium* seedlings, they do not influence the time of the first appearance of flowers but they do affect strongly the numbers of flowers produced (Olivera & Browning, 1993a). This suggests that, if GAs are involved in maintaining the juvenile, non-flowering state in *P. avium*, either those GAs are present in the seedling before the inhibitors are applied and continue to exert their influence in the presence of the inhibitor or the inhibitors do not affect the production of the active compounds i.e the active compounds are not GAs.

Further work is required to quantify accurately the 12α,13-dihydroxy GAs from germination through seedling development to flowering, and to determine the biosynthetic route to these GAs in developing fruits and whether they are biosynthesised by vegetative tissues.



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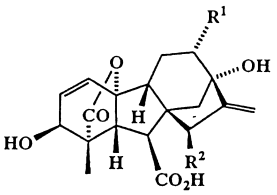
2 R^1 = OH, R^2 = H
3 R^1, R^2 = OH



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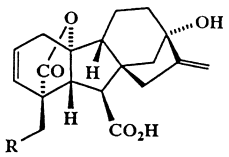

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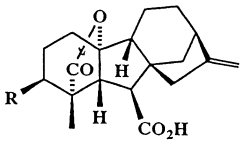

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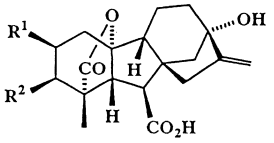

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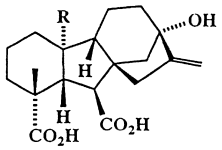
12 R^1, R^2 = H
13 R^1 = OH, R^2 = H



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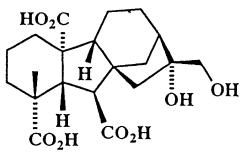

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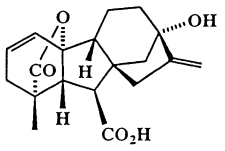
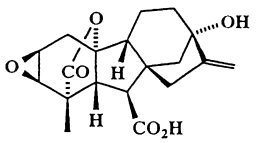
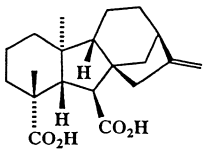


17



18



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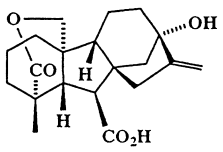
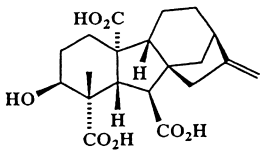


20



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3. Experimental

3.1. Plant material

Seeds were obtained from ripe fruits harvested from mature trees of the self-fertile sweet cherry cv. Stella, planted on Colt rootstocks (6 × 4 m or 4.5 × 3 m spacing) in 1982, and pruned minimally thereafter. Seeds were separated from the fruits, cleaned, dried and then stored in paper bags at 8°C until required for seedling production or GA extraction. Seeds were removed from the endocarp, soaked in well aerated water for 24 h and surface sterilised. A sample of 500 seeds was taken for GA analysis and the remaining seeds were stratified in moist sand for 16 weeks at 3°C. After stratification, the seeds were washed gently. Approximately 50% of these had germinated (radicle 5–10 mm long). A second sample of 500 seeds was taken for GA analysis to estimate any changes that had taken place through stratification. The remaining seeds (with radicle) were placed in a peat-based compost and transferred to glasshouse conditions (16 h light/8 h dark) for continued growth. Further samples, consisting of 500 whole seedlings and 500 shoot apices (expanding internodes and leaves) were taken during the first year of growth and subsequently apices were sampled from plants one, two or three years old and from mature orchard trees.

3.2. Plant tissue collection

Samples of plant tissue (seeds, seedlings or shoot apices) for GA analysis were collected from the orchard or glasshouse and immediately plunged into liquid nitrogen. These were then either extracted or freeze-dried and stored at −20°C until required.

3.3. Extraction and purification

The purification procedure was based on a method that avoided the use of solvent partitioning and thus ensured that polyhydroxylated GAs were not excluded from the analysis (Blake et al., 1993). Freeze-dried samples were homogenized in 80% MeOH (vol/vol) containing 20 mg l^{-1} BHT (10 ml solvent to 1 g dry weight tissue) and immediately filtered. In some experiments, 0.34 KBq of [1,2-^{3}H]GA_1 (1406 GBq $mmol^{-1}$; Du Pont de Nemours GmbH, NEN Division, Dreiech, Germany) was added before homogenization to estimate recoveries. The residue was re-extracted with MeOH (20 ml MeOH to 1 g tissue) overnight at 4°C and refiltered. MeOH was removed from the combined filtrates under reduced pressure on a rotary film evaporator (RFE) at 35°C, an equal volume of 0.5 M K–Pi buffer (pH 8.2) was added to the aqueous residue, the pH was adjusted to 8.0 (1 M KOH) and the extract was frozen and stored at −20°C. After thawing, the extract was centrifuged (33,000 × *g*; 15 min at 4°C), and the supernatant was added to a column (1.5 × 9 cm) of PVPP pre-equilibrated with 0.5 M K–Pi buffer (pH 8.2). The column was washed with a further 20 ml K–Pi buffer, and the combined eluates were adjusted to pH 3.0 (2 M HCL) and added to a column (1.5 × 18 cm) of charcoal:celite (1:2 wt/wt) {pre-washed with 80% (vol/vol) acetone (150 ml) and water (150 ml), pH 3.0}. The column was then washed with water (150 ml), pH 3.0, followed by 10% acetone (100 ml). GAs were eluted in 80% acetone (100 ml), the acetone was removed (RFE) from the eluate and the aqueous residue was adjusted to pH 8.0 and added to a column (2 × 6 cm) of QAE Sephadex A-25, pre-equilibrated with sodium formate (0.5 M), before washing with formic acid (0.2 M) and, finally, 0.02 M sodium formate. After loading, the column was washed with water (60 ml) pH 8.0 and the GAs were eluted with 0.2 M formic acid (80 ml). The eluate was fed directly through two pre-equilibrated C_{18} Sep–Pak cartridges in series, and after washing with water (10 ml), pH 3.0, the GAs were eluted with 80% (vol/vol) MeOH (10 ml), which was then evaporated to dryness (RFE) after adding 2 ml toluene.

GAs were purified further by reverse phase HPLC using a Nucleosil 50 ODS column 8 mm i.d. × 250 mm. The column was eluted at a flow rate of 2 ml min^{-1} with 10% (vol/vol) MeOH for 5 min, followed by a linear gradient to 100% (vol/vol) MeOH over 45 min (solvents contained 50μl l^{-1} acetic acid). The samples were dissolved in 10% MeOH (200 μl) and injected into the column using a 500 ml loop. Fifty fractions (2 ml) were collected and evaporated to dryness using a centrifugal vacuum concentrator (CVC). The extracts were re-dissolved in MeOH (500 μl) and aliquots (100 μl) were tested for GA-like activity using the lettuce (*Lactuca sativa* L. cv. Arctic King) hypocotyl bioassay (Frankland & Wareing, 1960). Fractions showing biological activity were then combined as appropriate, methylated with excess ethereal diazomethane, evaporated under a stream of O_2–free N_2 and dissolved in anhydrous dichloromethane (150 μl) for TLC. The fractions that did not show any GA-like activity were derivatised separately using the above procedures.

Extracts were applied in a narrow band to aluminium-backed silica gel coated plates (methanol prewashed). Methylated GA standards were applied within a scored zone close to each vertical outside edge of the plate and the plate was eluted with $CHCl_3$/MeOH (9:1 vol/vol). After development, vertical strips enclosing the GA standards were cut from the plate, sprayed with H_2SO_4–EtOH (1:20 vol/vol) and heated at 110°C for 10 min before the GAs were detected under UV light at 254 nm. Silica gel was removed

from the plates in broad zones corresponding to the Rf of appropriate GA standards, eluted with EtOH (3 × 1 ml) and the extract was evaporated to dryness (CVC). Each fraction was dissolved in 25 μl Tri–Sil BSA (Pierce and Warriner, Chester, UK), heated to 100°C for 5 min, evaporated to dryness and redissolved in 5 μl BSTFA (Pierce and Warriner, Chester, UK) to produce the trimethylsilyl ethers of the GA-methyl esters (MeTMSi) for GC–MS. Recoveries of [1,2-^{3}H]GA_1 after these procedures were 50–60%.

3.4. Extracts for bioassay

To quantify the GAs by bioassay, aliquot samples, equivalent to 100 seeds, 100 whole seedlings or 100 shoot tips were subjected to the lettuce hypocotyl bioassay (Frankland & Wareing, 1960). Each bioassay was performed on duplicate extracts of each tissue sample.

3.5. Capillary column GC–MS

Derivatised extracts (MeTMSi) were analysed by GC–MS, using a VG TRIO-1 MS system coupled to an HP 5890 Series II GC equipped with a split/splitless injector. The XTI 5 capillary column (Restek Corp. Bellafonte, PA., 30 m long × 0.25 mm i.d., 0.25 m df) was coupled directly to the ion source with an interface temperature of 285°C. Carrier gas (He) was supplied under electronic pressure control to maintain a linear flow rate of 40 cm s^{-1}. Samples (1 μl) were co-injected with a 'Parafilm' extract (0.1 μl), to allow the calculation of Kovats Retention Indices (KRI) (Gaskin & MacMillan, 1991) the injector (275°C) was operated in the splitless mode, at an oven temperature of 60°C with the split-valve (50:1) closed, and, after 1.0 min, the split-valve was opened and the oven temperature increased at 20°C min^{-1} to 220°C and then at 4°C min^{-1} to 300°C. Mass spectra were acquired by the VG Lab-Base data system commencing 14 min after injection, scanning from 50 to 700 amu at 0.9 s mass $decade^{-1}$. The electron energy was 70 eV and the source temperature was 200°C.

Selected Ion Monitoring (SIM) was used to search, at the appropriate KRI, for polar GAs in extracts of vegetative tissues from mature trees. The mass spectrometer was tuned to monitor the following ions: for GA_{85} (*m*/*z* 375, 491, 504, 579, 594), GA_{86} (*m*/*z* 339, 502, 592, 667, 682), GA_{87} (*m*/*z* 489, 502, 548, 577, 592) and GA_{32} (*m*/*z* 339, 500, 590, 665, 680). Also SIM was used to search for the GAs of the 13-hydroxylation pathway, which were not detected in extracts by full scan mass spectrometry. The following ions were monitored: for GA_{12} (*m*/*z* 240, 285, 300, 328, 360), GA_{44} (*m*/*z* 207, 238, 373, 417, 432) and GA_{53} (*m*/*z* 235, 241, 251, 389, 448).

3.6. Preparation of GA_{13}-trimethyl ester

An ethereal solution of diazomethane was added dropwise to a stirred solution of GA_{13} (1.00 g, 2.64 mmol) in MeOH (10 ml), until the yellow colour persisted. The solvent was removed under reduced pressure and the residue was chromatographed on silica gel using EtOAc:Hexane (1:2) as the solvent to give GA_{13}-7,19,20-trimethyl ester (911 mg) as a white foam; mp 117–119°C [lit. 121–122°C, (Galt, 1965)]; IR cm^{-1}: 1703 (*s*), 1725 (*s*), 3460 (*m*); ^{1}H NMR (300 MHz, $CDCl_3$); δ 0.92–1.09 (1H, *m*, H-11), 1.23 (3H, *s*, H-18), 1.23–2.32 (12H, *m*), 2.58 (1H, *d*, *J* = 3D 12.6 Hz, H-5), 2.58 (1H, *m*, H-13), 3.59 (3H, *s*, CO_2CH_3), 3.65 (3H, *s*, CO_2CH_3), 3.75 (3H, *s*, CO_2CH_3), 3.88 (1H, *d*, *J* = 3D 12.8 Hz, H-6), 3.97 (1H, *br s*, H-3), 4.79 (1H, *br s*, H-17), 4.88 (1H, *br s*, H-17); ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.6 (C-11), 22.6 (C-18), 28.9 (C-1), 30.0 (C-2), 31.5 (C-12), 36.1 (C-14), 39.3 (C-13), 46.0 (C-15), 49.2 (C-4), 49.9 (C-5), 50.0 (C-8), 50.6 (C-6), 50.9 (CO_2CH_3), 51.3 (CO_2CH_3), 51.4 (CO_2CH_3), 56.1 (C-9), 56.9 (C-10), 70.3 (C-3), 105.9 (C-17), 156.7 (C-16), 174.6, 175.2, 175.3 (C-7, C-19, C-20); MS (EI) *m*/*z* (rel. int): 420 $[M]^+$ (7), 388 (45), 360 (17), 328 (100), 310 (41), 300 (60), 282 (60), 268 (55), 241 (28), 223 (43); HRMS (EI) *m*/*z*: Calcd. for $[M]^+$ $C_{23}H_{32}O_7$: 420.2148; Found: 420.2154; Anal. Found: C, 65.45; H, 7.72. $C_{23}H_{32}O_7$ requires: C, 65.70; H, 7.67.

3.7. Preparation of GA_{25}-trimethyl ester

N,*N*-Thiocarbonyldiimidazole was added to a solution of the GA_{13} trimethyl ester (20 mg, 0.048 mmol) in dry CH_2Cl_2 (3 ml) under an atmosphere of N_2 (44.1 mg, 0.196 mmol) and the mixture was stirred under reflux for 16 h. The solvent was removed under reduced pressure and the residue was suspended in water. The product was extracted with EtOAc (×2), washed with water, with saturated NaCl, and dried over Na_2SO_4 to give an orange oil. Purification was carried out by flash chromatography on silica gel using EtOAc:Hexane (1:4) as the eluting solvent to give the 3-thiocarbonylimidazolate (23.5 mg) as a white powder; mp 50–52°C; IR cm^{-1}: 1262 (*s*), 1269 (*s*), 1386 (*m*), 1727 (*s*); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.17–2.60 (14H, *m*), 1.20 (3H, *s*, H-18), 2.61 (1H, *d*, *J* = 3D 12.5 Hz, H-5), 3.62 (3H, *s*, CO_2CH_3), 3.71 (6H, *s*, 2 × CO_2CH_3), 3.88 (1H, *d*, *J* = 3D 12.5 Hz, H-6), 4.84 (1H, *br s*, H-17), 4.90 (1H, *br s*, H-17), 5.87 (1H, *br s*, H-3), 7.08 (1H, *br s*, H-4′), 7.66 (1H, *br s*, H-3′), 8.40 (1H, *br s*, H-2′); ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.5 (C-11), 22.6 (C-18), 25.8 (C-1), 31.3 (C-2), 31.4 (C-12), 36.3 (C-14), 39.3 (C-13), 46.1 (C-15), 49.2 (C-4), 50.2 (C-8 + CO_2CH_3), 50.3 (C-5), 51.2 (CO_2CH_3), 51.5 (CO_2CH_3), 52.0 (C-5), 56.3 (C-9), 56.8 (C-10), 86.3 (C-3), 106.3 (C-17), 117.4 (C-3′), 131.0 (C-4′),

136.9 (C-2′), 156.0 (C-16), 173.6, 174.0, 174.9 (C-7, C-19, C-20), 182.7 (C-1′); MS (EI) *m*/*z* (rel. int): 530 $[M]^+$ (30), 403 (51), 371 (50), 343 (60), 311 (100), 283 (100), 251 (48), 223 (70), 181 (34), 129 (52); HRMS (EI) *m*/*z*: Calcd. for $[M]^+$ $C_{27}H_{34}N_2O_7S$: 530.2087; Found: 530.2088; Anal. Found: C, 61.00; H, 6.53; N, 4.96. $C_{27}H_{34}N_2O_7S$ requires: C, 61.11; H, 6.46; N, 5.28.

The imidazolate (631 mg, 1.19 mmol) was dissolved in dry benzene (70 ml) and heated to reflux under N_2 for 10 min. Tributyltin hydride (640 mg, 2.38 mmol) was added via syringe followed by azobisisobutyronitrile (40 mg, 10% wt). The resulting mixture was heated under reflux for a further 30 min, after which the solvent was removed under reduced pressure. The product was purified by flash chromatography on silica gel using EtOAc:Hexane (1:10) and the eluate was evaporated to dryness to give a yellow oil. Residual tin by-products were removed by washing with ammonia (×3) and the residue was chromatographed again to give GA_{25}-trimethyl ester (369 mg) as a white solid; mp 77–79°C; IR cm^{-1}: 1729 (*s*); ^1H NMR (300 MHz, $CDCl_3$): δ 1.00–2.58 (16H, *m*), 1.13 (3H, *s*, H-18), 2.13 (1H, *d*, *J* = 3D 12.6 Hz, H-5), 3.59 (3H, *s*, CO_2CH_3), 3.85 (3H, *s*, CO_2CH_3), 3.71 (3H, *s*, CO_2CH_3), 3.84 (1H, *d*, *J* = 3D 12.6 Hz, H-6), 4.81 (1H, *br s*, H-17), 4.89 (1H, *br s*, H-17′); ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.5 (C-11), 21.5 (C-2), 28.4 (C-18), 31.5 (C-12), 36.4 (C-14), 36.5 (C-1), 37.7 (C-3), 39.5 (C-13), 44.9 (C-4), 46.0 (C-15), 50.0 (C-8), 50.8 (C-5 + CO_2CH_3), 51.2 (CO_2CH_3), 51.3 (CO_2CH_3), 56.3 (C-6), 56.6 (C-9), 57.0 (C-10), 105.8 (C-17), 156.5 (C-16), 174.5, 175.6, 176.0 (C-7, C-19, C-20); MS (EI) *m*/*z* (rel. int): 404 $[M]^+$ (32), 374 (71), 372 (74), 342 (35), 328 (26), 312 (93), 284 (100), 255 (26), 225 (73), 183 (38); HRMS (EI) *m*/*z*: Calcd. for $[M]^+$ $C_{23}H_{32}O_6$: 404.2199; Found: 404.2190.

3.8. Preparation of 16α,17-dihydroxy-16,17-dihydro-GA$_{25}$-trimethyl ester

4-Methyl morpholine-*N*-oxide and a crystal of *p*-toluene-sulphonic acid were added to a solution of GA_{25} trimethyl ester (107 mg, 0.265 mmol) in acetone (8 ml) and water (1 ml). Osmium tetroxide (a few crystals) in *t*-butyl alcohol (1 ml) was added and the mixture was stirred at room temperature for 3 h. The reaction mixture was then diluted with water and extracted with EtOAc (×2). The organic extracts were combined and washed with water, with saturated NaCl, and dried over Na_2SO_4, and the solvent evaporated under reduced pressure to give the crude product as a brown oil (114 mg). Purification was carried out by flash chromatography on silica gel using EtOAc:Hexane (4:1), to yield a white crystalline solid which was recrystallised from ether/heptane to give pure 16α,17-dihydroxy-16,17-dihydroxy-GA_{25}-7,19,20-trimethyl ester; mp 47°C; IR cm^{-1}: 1143 (*m*), 1169 (*m*), 1198 (*m*), 1230 (*m*), 1729 (s), 2930 (*m*), 3437 (*v*); ^1H NMR (300 MHz, $CDCl_3$): δ 0.88–2.04 (15H, m), 1.11 (3H, *s*, H-18), 2.07 (1H, *d*, *J* = 3D 12.6 Hz, H-5), 2.43 (1H, *m*, H-15a), 3.58–3.76 (2H, *m*, H-17), 3.60 (3H, *s*, CO_2CH_3), 3.65 (3H, *s*, CO_2CH_3), 3.74 (3H, *s*, CO_2CH_3), 3.86 (1H, *d*, *J* =3D 12.6 Hz, H-6); ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.7 (C-11), 21.4 (C-2), 21.9 (C-12), 28.3 (C-18), 34.6 (C-14), 36.3 (C-1), 37.6 (C-3), 43.1 (C-13), 44.8 (C-4), 50.4 (C-8), 50.9 (CO_2CH_3), 51.3 (CO_2CH_3), 51.5 (C-15), 51.5 (CO_2CH_3), 51.7 (C-5), 56.5 (C-6), 57.0 (C-10), 58.1 (C-9), 66.8 (C-17), 82.2 (C-16), 174.3, 175.8, 176.0 (C-7, C-19, C-20); MS (EI) *m*/*z* (rel. int): 406 $[M - 32]^+$ (14), 375 (25), 347 (18), 315 (27), 287 (40), 245 (17), 227 (18), 185 (24), 129 (28), 105 (42); Anal. Found: C, 62.85; H, 8.02. $C_{23}H_{34}O_8$ requires: C, 63.00; H, 7.81.

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