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GIBBERELLINS AND RELATED COMPOUNDS IN YOUNG FRUITS OF PEA AND THEIR RELATIONSHIP TO FRUIT-SET

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Key Word Index—*Pisum sativum*; Leguminosae; pea; fruit; fruit-set; gibberellins.

Abstract—Six fractions with the capacity to induce fruit-set in pea (*Pisum sativum*) were obtained after purified extracts of young pea fruits (4–6 days after anthesis) were separated by reverse-phase HPLC. Two of these also had gibberellin (GA)-like activity in the dwarf-rice cv. Tan-ginbozu bioassay. The HPLC fractions were shown by full-scan GC–MS to contain the GAs previously identified in pea (GA_1, GA_3, GA_8, GA_{17}, GA_{19}, GA_{29}, GA_{81} and GA_{29}-catabolite) and, in addition, GA_{13}, GA_{43}, GA_{60}, *iso*-GA_3, *iso*-GA_7 open-lactone, GA_8-catabolite, 2β-hydroxy GA_{60}(GA_{91}) and the 16,17-dihydrodihydroxylated derivatives of GA_4, GA_7, GA_9, GA_{12}, GA_{34} and *ent*-kaurenoic acid. Indole-3-acetic acid was also identified. Gibberellin A_1 and GA_3 were detected in the HPLC fractions with the highest fruit-set activity. A later-eluting fraction, which also had very high fruit-setting capacity, but did not show any GA-like activity, was further fractionated by normal-phase HPLC into two peaks of fruit-set activity. However, no previously-characterized candidates for the active components were detected in either fraction by GC–MS analysis. These results provide further evidence for the involvement of GAs, particularly GA_1 and GA_3, in the control of fruit-set and growth in pea and suggest that other, as yet unidentified factors are also involved in this process.

INTRODUCTION

There is a general assumption that fruit-set and fruit growth are under hormonal control [1]. Evidence for this comes mainly from observations that parthenocarpic fruit development can be induced in a range of plant species by treatment with different plant growth substances, which thereby substitute for the presence of fertilized ovules [1, 2]. In addition, developing fruits and, particularly, immature seeds are rich sources of auxins [3, 4], gibberellins (GAs) [5, 6] and cytokinins [7] and, in some cases, correlations between fruit growth and the levels of endogenous hormones have been found [4, 8, 9]. However, despite this circumstantial evidence, there is still no direct indication that fruit growth is regulated by any of the major groups of hormones.

Some of the strongest evidence for the involvement of GAs in fruit development has been obtained in pea (*Pisum sativum*). Although parthenocarpic development of pea fruits may be obtained by applying GAs, auxins or cytokinins to seedless ovaries [9–12], only GAs produce fruits with similar shape and size to pollinated fruits. Also, inhibitors of GA biosynthesis reduce the growth of pollinated pea ovaries and this is restored by simultaneous application of GA_3 [13, 14, and Santes, C. M. and García-Martínez, J. L., unpublished results]. GC–MS analyses have shown that immature pea seeds contain a wide spectrum of GAs [15 (and references therein)]. GA_1, GA_3, GA_8, GA_{19}, GA_{20} and GA_{29} have been identified in ovules and pods from 4-day-old pollinated ovaries of pea cv. Alaska, and GA_{17}, GA_{81} and GA_{29}-catabolite were identified additionally in pods [13, 16]. Furthermore, the highest concentrations of GA_1 and GA_3 in pollinated ovaries correlated with the time of rapid pod elongation [16], suggesting that these GAs may control pod development.

It was decided to examine in detail the involvement of endogenous GAs and other factors in fruit development using the capacity to set emasculated pea flowers as a bioassay. Modern procedures, such as gas chromatography–mass spectrometry (GC–MS) and immunoassay, have largely replaced bioassays for hormone analysis, but such assays are still necessary for the detection of uncharacterized active compounds. Although many bioassays have been described for GAs [17], there are few reports on the assay of fruit-set activity in extracts from fruits or seeds [10, 18, 19]. The main objective of the present work was to characterize, by full-scan GC–MS, compounds in young pea fruits with fruit-set activity.

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RESULTS AND DISCUSSION

Hormonal activity in reverse-phase HPLC fraction

A total of 2427 pollinated pea ovaries (412 mg ovary^{-1}) at the time of maximal pod growth rate (day 4 to day 6) were extracted and purified as described in the Experimental. After reverse-phase HPLC, 40 fractions were collected and their fruit-set activity investigated by applying aliquots equivalent to 10 and 30 g fr. wt to day O ovaries from emasculated pea flowers. Activity was detected only between fractions 12 and 29 and, considering the results obtained from the two aliquots, it was localized principally in the following six groups of fractions: 12, 14–15, 18, 20, 25–26 and 28–29 (Fig. 1).

In order to determine whether or not the detected fruit-set activities were due to GA-like compounds, two aliquots from each HPLC fraction, equivalent to 33 and 100 g fr.wt, were bioassayed on seedlings of dwarf-rice cv. Tan-ginbozu. Activity was detected in fractions 5–6, 8–9, 13–15 (same retention time as GA_1), 23 (same retention time as GA_{20}), 25–27 (same retention time as GA_{19}) and 35 (Fig. 2). By comparing the results of both bioassays, it can be observed that fractions 14–15 and 25–26 were active in both fruit-set and dwarf-rice bioassays. Fractions 12, 18, 20 and 28–29 were active in fruit-set, but not in the dwarf-rice bioassay and may contain GAs with very low stem-extension activity or non-GA compounds with fruit-set activity. Fractions 5–6, 8–9, 23 and 35, showed GA-like activity (Fig. 2) without inducing fruit-set of pea ovaries (Fig. 1).

Qualitative analyses of reverse-phase HPLC fractions by GC–MS

Reverse-phase HPLC fractions were combined according to their activity in both bioassays and analysed by full-scan capillary GC–MS. The presence of the following GAs in the combined fractions was indicated by comparison of their mass spectra and Kovat's retention indices (KRIs) with published data [20] (Table 1): GA_1, GA_1 1,10-ene diacid, GA_3, *iso*-GA_3, GA_8, GA_8-catabolite, GA_{13}, GA_{17}, GA_{19}, GA_{20}, GA_{29}, GA_{29}-catabolite, GA_{43}, GA_{60}, GA_{81}, GA_{91} (2β-hydroxyGA_{60}), *iso*-GA_7 open-lactone, 11α-hydroxyGA_{14} (putative), 12α-hydroxyGA_{53} (putative) and the 16α,17-dihydro-16α,17-dihydroxy derivatives of GA_4, GA_7 (putative), GA_9, GA_{12} and GA_{34}. In addition, *ent*-6α,7α-dihydroxykaurenoic acid, *ent*-16β,17-dihydroxykauranoic acid, *ent*-6α,7α,16α,17-tetrahydroxykauranoic acid and IAA were identified. The structures of the hydroxylated derivatives of GAs and *ent*-kaurenoic acid are shown in Fig. 3. Slight differences in KRIs between sample and published values would be expected because of differences in the GC conditions and film thicknessess of the columns used. However, in the case of GA_9 16,17-dihydrodiol, the discrepancy is sufficiently large to throw doubt on the stereochemical assignment at C-16.

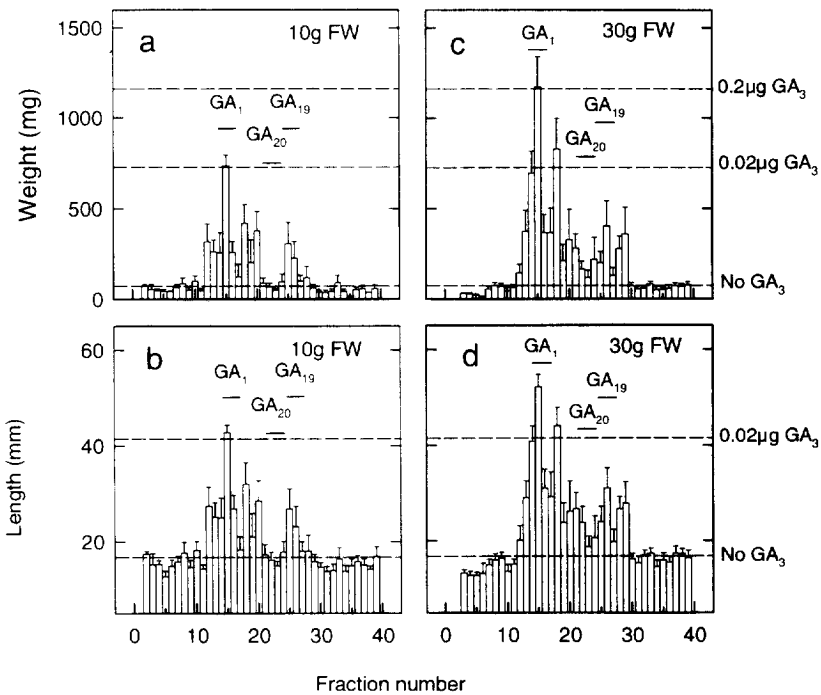


Fig. 1. Fruit-set activity in fractions after reverse-phase HPLC of extracts of young pea fruits. Aliquots equivalent to 10 g (a, b) and 30 g (c, d) FW from day 4 to day 6 pollinated ovaries (2427 ovaries; 412 mg ovary^{-1}) were bioassayed. Weight (a, c) and length (b, d) of ovaries were measured 6 days after treatment. Dashed lines show the response of ovaries to known amounts of GA_3. Elution times of standard GAs in the HPLC gradient employed are indicated. Values are means of six replicates ± s.e.m.

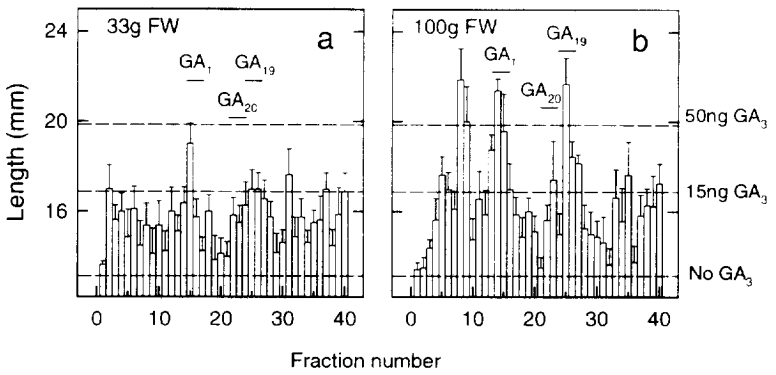


Fig. 2. GA-like activity in fractions after reverse-phase HPLC of extracts from young pea fruits. Aliquots equivalent to 33 g (a) and 100 g (b) FW from day 4 to day 6 pollinated fruits (2427 ovaries; 412 mg ovary^{-1}) were bioassayed. Length of second leaf sheath of rice seedlings was measured 7 days after treatment. Dashed lines show the response of rice seedlings to known amounts of GA_3. Elution times of standard GAs in the HPLC gradient employed are indicated. Values are means of eight replicates ± s.e.m.

Fractions 13–15, containing GA_1 and GA_3, had the highest fruit-set activity (Fig. 1) and were among the most active fractions in the dwarf-rice bioassay (Fig. 2). This is in accordance with the very high activity of GA_1 and GA_3 in most bioassays for GAs [17, 21] and for the induction of parthenocarpic fruit development in pea [12, 13]. On the basis of the correlation between GA_1 and GA_3 contents and fruit growth, it has been suggested previously that GA_1 and/or GA_3 are the endogenous regulators of fruit-set and pod development in pea [16]. The *iso*-GA_3 in fractions 13–15 (Table 1) is assumed to be an artefact produced by isomerization of GA_3 during the extraction and purification procedures.

The very low fruit-set activity in fractions 22–23 (Fig. 1), which contained GA_{20} (Table 1), compared with that in fraction 13–15, is in accordance with GA_{20} being less active than GA_1 and GA_3 in inducing fruit-set when applied to unpollinated pea ovaries [13]. Gibberellin A_{20} is thought not to be active *per se* in fruit-set [22] but, as has been shown for pea shoot extension [23], may require conversion to GA_1 or GA_3 for activity. However, fractions 22–23 also contained less activity than fractions 13–15 in the dwarf-rice bioassay, despite the fact that Tan-ginbozu rice seedlings respond about equally to GA_{20} and GA_1 [21]. Since endogenous levels of GA_{20} in pea ovaries at the time of harvest (day 4–day 6) are *ca* 1.5-fold higher than those of GA_1 and GA_3 together [16], it is possible that some inhibitor(s) may be present in fractions 22–23. The fruit-set activity of fractions 25–27 may be due to GA_{19}, which presumably is also not active *per se*, but as a consequence of its conversion to active GAs.

Further purification and analysis of fruit-setting factors in fraction 18

Fraction 18 from reverse-phase HPLC was of considerable interest because it contained high activity in the fruit-set bioassay (Fig. 1), while showing no activity in the dwarf-rice bioassay (Fig. 2). This fraction contained GA_9 dihydrodiol (Table 1). In order to purify further the active components in fraction 18, a new extract from 2849 pollinated day 4 to day 6 pea ovaries (527 mg ovary^{-1}) was obtained, which showed bioassay activities after reverse-phase HPLC similar to those presented in Figs 1 and 2. Fraction 18 was then fractionated further by normal-phase HPLC, collecting 60 fractions of 2 ml each. Different aliquots from the HPLC fractions were used in each bioassay (equivalent to 40 g fr. wt for fruit-set and to 100 g fr. wt for the dwarf-rice bioassay). Activity in the fruit-set bioassay was located in two peaks corresponding to fractions 36–37 (which showed very high activity) and fractions 43–45 (much less active) (Fig. 4). No activity was found when fractions 30–48 were analysed by the dwarf-rice bioassay (Fig. 5).

Fractions 35–37 and 42–46 were analysed individually by full-scan capillary GC–MS. No candidates for the active substance could be detected in the active fractions (36, 37, 43–45), whereas GA_9 dihydrodiol was detected in fraction 46, which did not show fruit-set activity (Fig. 4). Studies to establish the identity of the fruit-setting factors in the active fractions will be continued. On the basis of the procedure used to purify these active components, they would appear to be weak acids with properties similar to those of the GAs. Potentially active compounds, such as the auxin 4-Cl-IAA, identified previously in pea seeds [4, 24], or cytokinins, would be removed during purification and thus can be discounted. The identification of IAA in fractions 16–17 (Table 1) confirms the presence of this auxin in young pea fruits [4]. The application of IAA to unpollinated pea ovaries does not induce parthenocarpic development [10] and, therefore, the low fruit-set activity in fractions 16–17, is probably due to the presence of traces of GA_1 (Table 1).

A total of 28 GAs and related compounds were detected in the young pea fruits. These include several GAs that have not been previously reported for this tissue. GA_{60} and its 2β-hydroxylated derivative, which has been

Table 1. Gibberellins and related compounds identified in reverse-phase HPLC fractions from purified extracts of young pea fruits (day 4–day 6) (identification was by full-scan GC–MS on the basis of a comparison of the mass spectra and KRIs of MeTMSi derivatives with those of authentic standards)

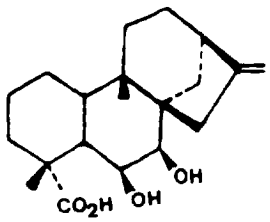
Compound	Fractions	KRI	Diagnostic ions (m/z) with % relative intensity in reference (Ref.) and sample							
GA_1			Ion	$[M]^+$506	491	448	376	375	235	207	180
	13–15	2668	Ref.	100	9	18	14	9	6	24	8
	16–17	2676	Sample	100	13	20	21	12	14	26	27
GA_3			Ion	$[M]^+$504	489	475	445	370	297	238	208
		2692	Ref.	100	9	12	12	24	14	21	37
	13–15	2699	Sample	100	8	9	9	14	19	23	47
GA_8			Ion	$[M]^+$594	579	535	448	379	375	238	207
		2818	Ref.	100	4	6	14	7	5	13	18
	10–12	2821	Sample	100	8	9	24	13	13	22	39
GA_{13}	22–23		Ion	$[M]^+$492	477	460	436	400	310	160	129
	24	2596	Ref.	1	8	13	16	35	38	29	100
	25–27	2592	Sample	1	8	12	14	33	41	33	100
GA_{17}			Ion	$[M]^+$492	463	460	433	401	373	251	208
		2575	Ref.	43	11	23	26	10	23	24	100
	25–27	2579	Sample	26	6	24	22	12	21	18	100
GA_{19}			Ion	$[M]^+$462	434	402	374	345	285	239	207
		2596	Ref.	7	100	37	64	23	21	33	23
	25–27	2601	Sample	5	100	40	69	36	35	47	36
GA_{20}			Ion	$[M]^+$418	403	375	359	301	235	207	193
		2482	Ref.	100	16	46	12	12	8	30	9
	22–23	2492	Sample	100	16	61	18	16	9	43	12
GA_{29}			Ion	$[M]^+$506	491	447	389	375	303	235	207
		2684	Ref.	100	11	6	8	15	20	10	39
	10–12	2690	Sample	100	16	11	14	18	43	14	63
GA_{43}			Ion	$[M]^+$580	505	431	371	349	217	188	173
		2723	Ref.	9	6	100	15	24	57	61	56
	19–20	2722	Sample	7	6	100	19	20	38	31	52
GA_{60}			Ion	$[M]^+$506	491	477	447	375			
		2575	Ref.	100	13	10	26	92			
	10–12	2580	Sample	100	10	16	18	82			
GA_{81}			Ion	$[M]^+$506	491	459	447	389	375	303	207
		2673	Ref.	100	8	11	8	11	26	22	27
	10–12	2682	Sample	100	9	12	11	11	33	62	33
GA_1 1,10-ene diacid*			Ion	$[M]^+$520	460	430	398	370	311	281	221
		2576	Ref.	50	18	68	63	100	43	28	47
	16–17	2578	Sample	25	11	55	59	100	48	68	54
iso-GA_3*			Ion	$[M]^+$504	489	475	445	370	238	207	193
	10–12	2640	Ref.	100	10	13	9	13	27	11	12
	13–15	2633	Sample	100	10	21	16	15	23	19	12
16α,17-H_2 $(OH)_2$ GA_4*			Ion	$[M]^+$596	581	506	493	433	359	299	
		2926	Ref.	1	2	3	100	4	5	13	
	13–15	2932	Sample	1	3	1	100	7	5	10	
16α,17-H_2 $(OH)_2$ GA_7 (putative)*	8–9		Ion	$[M]^+$594	579	504	491	459	401	297	179
	10–12	2957	Sample	1	2	2	100	4	9	6	18
	13–15										
iso-GA_7 open lactone*			Ion	$[M]^+$520	461	430	371	332	311	281	273
		2575	Ref.	3	2	4	7	100	27	20	76
	25–27	2573	Sample	3	2	7	12	100	38	34	66

Table 1. *Continued*

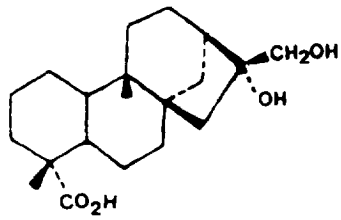
Compound	Fractions	KRI	Diagnostic ions (m/z) with % relative intensity in reference (Ref.) and sample							
GA_8-catabolite			Ion	$[M]^+$534	519	475	459	385	346	287	238
		2705	Ref.	75	14	12	13	8	9	9	100
	10–12	2713	Sample	44	12	13	13	12	11	11	100
16ξ,17-H_2 $(OH)_2$ GA_9*			Ion	$[M]^+$508	493	405	345	301	255	227	185
		2771	Ref.	1	3	100	6	7	7	7	5
	18	2788	Sample	1	2	100	5	7	7	7	13
11α-OH GA_{14} (putative)			Ion	$[M]^+$536	476	446	347	343	257	167	129
	16–17	2601	Sample	27	14	22	34	37	74	99	100
16α, 17-H_2 $(OH)_2$ GA_{12}*			Ion	$[M]^+$538	523	435	403	375	315	225	
		2789	Ref.	1	1	100	6	78	11	5	
	25–27	2789	Sample	1	1	100	4	66	8	6	
GA_{29}-catabolite			Ion	$[M]^+$446	431	429	417	414	387	327	238
		2673	Ref.	100	13	9	29	9	34	12	22
	16–17	2676	Sample	100	15	13	38	18	58	31	26
16α,17-H_2 $(OH)_2$ GA_{34}*			Ion	$[M]^+$684	669	581	431	315			
		3033	Ref.	9	2	100	4	3			
	10–12	3036	Sample	9	2	100	5	5			
12α-OH GA_{53} (putative)			Ion	$[M]^+$536	521	493	433	419	373	295	255
	16–17	2596	Sample	26	11	47	100	23	11	44	44
2β-OH GA_{60} (GA_{91})			Ion	$[M]^+$594	579	535	504	375	303	207	194
		2736	Ref.	100	8	9	4	21	32	19	29
	8–9	2738	Sample	100	10	17	9	36	58	70	66
ent-16β,17-$(OH)_2$ KA*			Ion	479	404	391	347	301	255	241	117
	31–32	2796	Ref.	1	1	100	5	9	6	17	61
	33–34	2792	Sample	2	1	100	7	7	7	13	92
ent-6α,7α-$(OH)_2$ KA*			Ion	$[M]^+$492	477	402	327	269	253	209	147
	33–34	2588	Ref.	1	60	24	4	100	12	28	18
	35	2581	Sample	1	65	24	6	100	9	28	13
ent-6α,7α,16α,17 -$(OH)_4$ KA*			Ion	655	567	477	417	387	309	269	209
		2970	Ref.	3	32	100	4	9	4	5	4
	22–23	2977	Sample	3	28	100	9	15	4	12	10
IAA			Ion	$[M]^+$261	218	202	189	130	89		
		1860	Ref.	37	1	100	2	5	2		
	16–17	1869	Sample	47	2	100	3	12	8		

*Abbreviations: 16α,17-$H_2(OH)_2GA_X$, 16α,17-dihydro-16α,17-dihydroxyGA_X (16ξ,17-$H_2(OH)_2GA_9$, stereochemistry at C-16 is uncertain); *ent*-16β,17-$(OH)_2$KA, *ent*-16β,17-dihydroxykauranoic acid; *ent*-6α,7α-$(OH)_2$KA, *ent*-6α,7α-dihydroxykaurenoic acid; *ent*-6α,7α,16α,17-$(OH)_4$KA, *ent*-6α,7α,16α,17-tetrahydroxykauranoic acid; GA_1 1,10-ene diacid, *ent*-3α,13-dihydroxy-20-norgibberella-1(10),16-diene-7,19-dioic acid; *iso*-GA_3, *ent*-2β,3α,13-trihydroxy-20-norgibberella-1(10),16-diene-7,19-dioic acid 19,2-lactone; *iso*-GA_7 open lactone, *ent*-2β,3α-dihydroxy-20-norgibberella-1(10),16-diene-7,19-dioic acid.

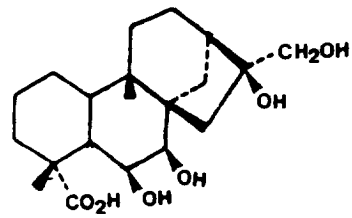
designated GA_{91} [25], are conceivably produced via GA_{20}. There is also a series of non-13-hydroxylated GAs, including GA_{13}, GA_{43} and the dihydrodiols of GA_4, GA_7, GA_9, GA_{12} and GA_{34}. The dihydrodiols of GA_4 [26, 27], GA_9 [28] and GA_{12} [29] have been described previously in other species. The identity of the GA_7 derivative is assumed from its mass spectrum and must still be considered putative. This compound was shown previously by GC–MS to be present in high concentrations in pea ovules, although no structure was suggested at that time [17]. Since none of the parent compounds for these diols could be detected in the young fruit, it appears that these GAs are being turned over very rapidly. The large amounts of GA_7 dihydrodiol and the presence of *iso*-GA_7 open lactone indicate that GA_7 formation is a major pathway in young pea fruit, probably taking place in the ovules [16]. It is possible that GA_7 is also converted to GA_3 by 13-hydroxylation, al-



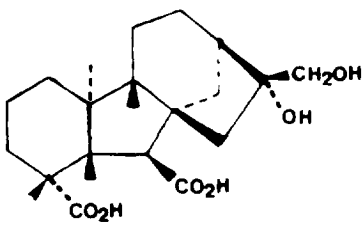
ent-6α. 7α-$(OH)_2$ kaurenoic acid



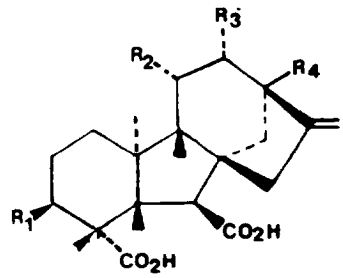
ent-16ß. 17-$(OH)_2$ kauranoic acid



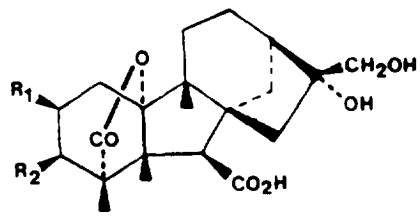
ent-6α. 7α. 16α. 17-$(OH)_4$ kauranoic acid



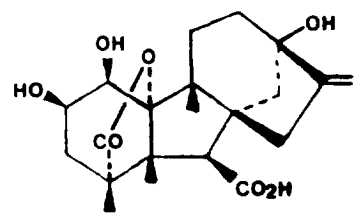
16α. 17-$H_2(OH)_2$ GA_{12}



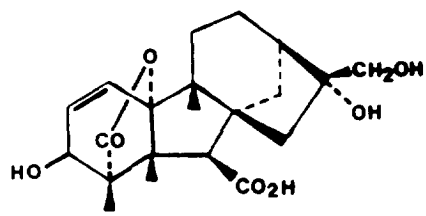
R_1	R_2	R_3	R_4	
OH	OH	H	H	11α-OH GA_{14}
H	H	OH	OH	12α-OH GA_{53}



R_1	R_2	
H	OH	16α. 17-$H_2(OH)_2$ GA_4
H	H	16α. 17-$H_2(OH)_2$ GA_9
OH	OH	16α. 17-$H_2(OH)_2$ GA_{34}



2ß-OH GA_{60} (GA_{91})



16α.17-$H_2(OH)_2$ GA_7

Fig. 3. Hydroxylated derivatives of *ent*-kaurenoic acid and GAs identified in day 4 to day 6 pea fruits (for abbreviations, see Table 1).

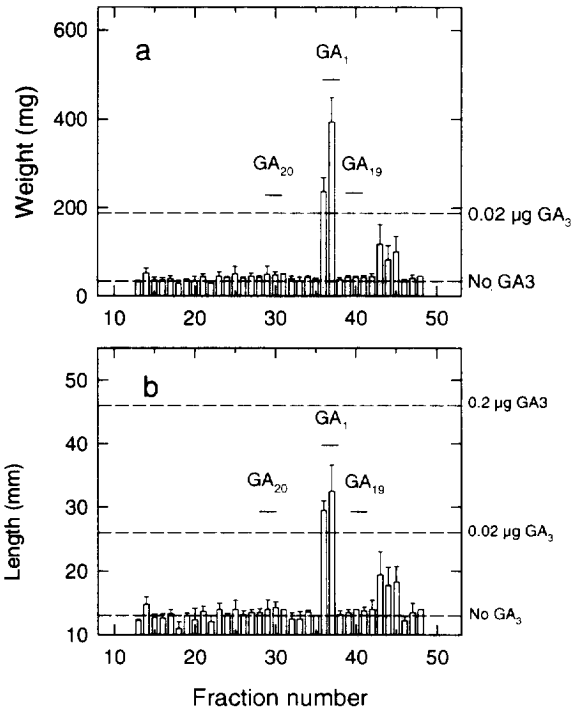


Fig. 4. Fruit-set activity in fractions after normal-phase HPLC of fraction 18, obtained after reverse-phase HPLC of extracts of young pea fruits (see Fig. 1). Aliquots equivalent to 40 g FW from day 4 to day 6 pollinated ovaries (2849 ovaries; 527 mg ovary^{-1}) were bioassayed as for Fig. 1. Weight (a) and length (b) of ovaries are shown.

though, in maize shoots, GA_3 is formed from GA_{20} via GA_5 [30]. Nothing is known about the formation of the 16,17-dihydrodiols, but they are presumably formed by hydration of the 16,17-epoxides. The biological activity of the 16,17-dihydrodiols has not been determined and it is not possible to draw any conclusions from the present work because of the complex composition of the fractions assayed. Although GA_9 dihydrodiol is clearly inactive, this may be due to the failure of the assay systems to 3β-hydroxylate this compound rather than to an intrinsic lack of biological activity of 16,17-dihydrodiols.

In conclusion, although GAs are responsible, in part, for the endogenous fruit-setting activity of young pea fruit, other important active factors remain to be identified.

EXPERIMENTAL

Plant material. A line of pea (*Pisum sativum* L., line V1) multiplied from seeds of cv. Alaska purchased originally from Asgrow (Complejo Agrícola de Semillas, Madrid, Spain), were used in expts. Plants were grown in vermiculite in a greenhouse as described previously [11] at 22° (day) and 17° (night), with a 16 hr photoperiod. Natural light was supplemented with sodium halide lamps (*ca* 93 μmol m^{-2} s^{-1} PAR at the level of the pot). Self-pollinated flowers were tagged on the day of anthesis (day 0).

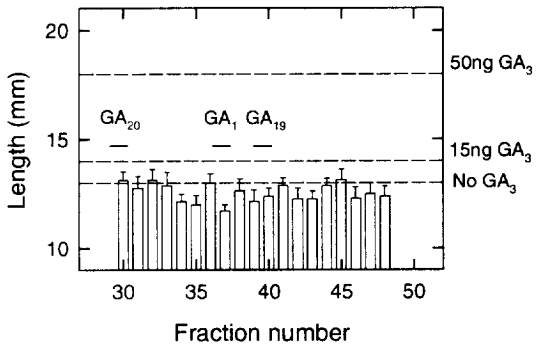


Fig. 5. GA-like activity in fractions after normal-phase HPLC of fraction 18, obtained after reverse-phase HPLC of extracts of young pea fruits (see Fig. 1). Aliquots equivalent to 100 g of FW from day 4 to day 6 pollinated ovaries (2849 ovaries; 527 mg ovary^{-1}) were bioassayed as in Fig. 2.

Pollinated ovaries were harvested between 4 and 6 days after anthesis (day 4 to day 6). Emasculation of the flowers was carried out 2 days before anthesis (day 2). Only the first flower of each plant was used for bioassays, all other flowers were removed as they appeared. Material was frozen in liquid N_2 and stored at −65° until extracted.

Extraction and purification of GAs and related compounds. Two independent harvests of pollinated pea ovaries were collected between day 4 and day 6, one of 2427 ovaries (412 mg ovary^{-1}), the other of 2849 ovaries (527 mg ovary^{-1}). Frozen material was finely ground in a mortar with liquid N_2 and then homogenized in 80% aq. MeOH (1:5, w/v), stirred overnight at 4° and re-extracted ×2 with MeOH (1:5, w/v). In order to check recoveries during extraction and purification, and to allow GAs to be located after HPLC, the following radioactive GAs were added to the homogenized samples: [$^3H_2$]GA_1 (1.39 TBq mmol^{-1}; Amersham International, Bucks, U.K.), [$^3H_4$]16,17-dihydro GA_{19} (2.45 TBq mmol^{-1}; prepared from GA_{19} by catalytic exchange by Amersham International) and [$^3H_2$]GA_{20} (1.41 TBq mmol^{-1}; from Prof. J. MacMillan, University of Bristol, U.K.). MeOH was removed *in vacuo* and the aq. residue purified by solvent partition, QAE-Sephadex A-25 chromatography, and C_{18}-SeqPak, following the procedure described previously [16].

Reverse-phase HPLC. Dried samples were dissolved in 28% aq. MeOH and injected onto a 10 μm μBondapak C_{18} column (30 cm long × 0.78 cm i.d.). Elution was carried out with a linear gradient of 28–100% aq. MeOH containing 50 μl l^{-1} HOAc, over 40 min at 2.5 ml min^{-1}. R_ts values for standards were: 15–16 min for GA_1, 22–23 min for GA_{20}, and 25–26 min for dihydroGA_{19}. Forty frs of 2.5 ml were collected to determine biological activity and for GC–MS.

Normal-phase HPLC. Fr. 18, after reverse-phase HPLC, was further purified by normal-phase HPLC. The dried residue was redissolved in hexane and injected onto a 5 μm Nucleosil 100 NO_2 column (12 cm long × 0.46 cm i.d.). The elution method, based on that

described in ref. [31], consisted of a linear gradient of *n*-heptane half-satd with 1 M HCO_2H to EtOAc containing 1% H_2O and 0.5% HCO_2H, over 60 min at 2 ml min^{-1}. Under these conditions, R_ts values for standards were: 29–30 min for GA_{20}, 36–37 min for GA_1, and 39–40 min for dihydroGA_{19}. Frs (2 ml) were collected between 13 and 48 min to detect biological activity and for GC–MS.

Bioassays. Hormonal activity in HPLC frs was determined using two different bioassays.

(a) *Dwarf-rice bioassay* [32]. Seeds of dwarf rice cv. Tan-ginbozu were germinated in H_2O for 2 days in darkness and then placed in tubes (16 cm long × 2.2 cm diam.) (8 seeds per tube), containing 4 ml of 1% agar. Aliquots of HPLC frs were dried, dissolved in H_2O (1 ml) and added to the rice culture medium. Tubes were covered with glass caps and sealed with Parafilm. After 7 days at 32° under continuous light from fluorescent tubes (130 μmol m^{-2} s^{-1} PAR), the length of the 2nd leaf sheath of each seedling was measured. Standard solns of GA_3 (0, 15 and 50 ng per tube) were also bioassayed.

(b) *Fruit-set bioassay.* Aliquots of HPLC frs were applied in 10% EtOH in 0.05% Tween 80 (20 μl $ovary^{-1}$) to day O ovaries from emasculated flowers cv. Alaska. A total of 6 ovaries per HPLC fr. were treated. Plants were grown in the greenhouse at 22° (day) and 17° (night) with a 16 hr photoperiod. Six days after treatment the lengths and wts of the pods were measured. GA_3 at 0, 0.02 and 0.2 μg $ovary^{-1}$ was also bioassayed.

Qualitative analysis by GC–MS. HPLC frs were combd according to their activity in the bioassays and taken to dryness *in vacuo*. Dried samples were methylated with CH_2N_2–Et_2O and then trimethylsilylated with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide at 90° for 30 min. Derivatized samples were analysed by full-scan capillary GC–MS on a OV-1 column (25 m × 0.2 mm i.d. × 0.25 μm) as described elsewhere [20]. A mixt. of *n*-alkanes obtained from a soln of Parafilm in hexane was co-injected for determining KRIs [33]. Identifications were based on comparisons of KRIs and MS with those of standards.

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