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GC/MS ANALYSIS OF THE PLANT HORMONES IN SEEDS OF *CUCURBITA MAXIMA*

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Key Word Index—*Cucurbita maxima*; Cucurbitaceae; endosperm; embryo; GC/MS; 12α-hydroxygibberellins; *ent*-12α-hydroxykaurenoic acids.

Abstract—The GC/MS detection is reported of over 30 compounds, in extracts of the endosperm and embryos from seeds of *Cucurbita maxima*. The compounds which were identified from reference spectra include: *cis,trans*-ABA; *trans,trans*-ABA; dihydrophaseic acid; IAA; GA_4; GA_{12}; GA_{13}; GA_{25}; GA_{39}; GA_{43}; GA_{49}; *ent*-13-hydroxy-, *ent*-6α,7α- and *ent*-7α,13-dihydroxy-, and *ent*-6α,7α,13-trihydroxykaur-16-en-19-oic acids; *ent*-7α,16,17-trihydroxy- and *ent*-6α,7α,16,17-tetrahydroxy-kauran-19-oic acids, *ent*-6,7-seco-7-oxokauren-6,19-dioic acid and/or *ent*-6,7-secokauren-6,7,19-trioic acid, and 7β,12α-dihydroxykaurenolide. New compounds, the structures of which were deduced from GC/MS data, include: the 12α-hydroxy-derivatives of GA_{12}, GA_{14}, GA_{37} and GA_4, and the 12β-hydroxy-derivatives of *ent*-7α-hydroxy- and *ent*-6α,7α-dihydroxykaurenoic acids.

INTRODUCTION

Biosynthetic pathways to C_{20}-gibberellins (GAs), C_{19}-GAs and *ent*-kaurenoids have been delineated in considerable detail in cell-free enzyme preparations from the endosperm of seed of *Cucurbita maxima* [1–3]. Complementary to these biosynthetic studies, concurrent investigations have been conducted on the endogenous compounds in these seeds. The results are reported in this and the accompanying three papers [4–6]. They provide an example of current methodology by which novel constituents are detected in extracts by GC/MS and identified with the further aid of microbiological conversion of known compounds in cultures of *Gibberella fujikuroi* (this paper and ref. [4]). Two of the new compounds were subsequently isolated in pure form and chemically characterized [5] and the results of all the investigations were used to identify a series of new products obtained with cell-free preparations from *C. maxima* [6].

This paper describes the extraction and identification by GC/MS of several compounds from *C. maxima* seeds at different stages of maturity. Different stages were used to ensure a broad spectrum of constituents and to find the most advantageous conditions for the isolation of new compounds. The experiments were not designed to permit a detailed comparison between the stages and thus do not justify conclusions with respect to changes in composition during seed development. This follows from the inherent variability of the fruits—pumpkin cultivars are not pure breeds—and the fact that a different fruit had to be used for each stage. Furthermore, no effort was made to account for losses during the preparation of samples and the final quantitation was only approximate.

RESULTS

Three series of experiments were done. Four fruits of *C. maxima* with seeds of maturity index 39, 61, 74 and 100 (see Experimental) were selected for the first series. Endosperm and embryos were separated, homogenized and extracted. One half of each homogenate was hydrolysed with cellulase before extraction. All extracts were derivatized and the Me and/or MeTMSi derivatives were analysed by GC/MS.

The results from the first series of experiments are shown in Table 1. Compounds **1–7** and **10–17** were identified by direct comparison of the mass spectra with reference spectra of the known compounds. Gibberellin A_{49} (**9**) was identified by mass spectra and GC retention time comparison (see Experimental) with authentic samples of GA_{48} (**8**) and GA_{49} (**9**). Compounds **18**, **19**, **21** and **23–25** were identified by comparison with the metabolites, obtained by microbiological conversion of *ent*-12α- and -12β-hydroxykaurenoic acids in cultures of *G. fujikuroi*, as described in the following paper [4]. The structures of compounds **20**, **26** and **27** were tentatively assigned from the mass spectral fragmentation patterns. Several unidentified compounds were also detected. Three of them may be GA_8, the 1(10),16-diene-diacid from 12α-hydroxyGA_4 (GA_{58}), and 2β-hydroxyGA_{39} 20,12-lactone; these latter three compounds were detected in the extracts from 61% endosperm and 74% endosperm, before and after treatment with cellulase.

In the second series of experiments, derivatized extracts from the endosperm and embryos of seeds of maturity index 40, 45 and 56, were also examined. The results qualitatively confirmed those presented in Table 1. In addition GA_4 (**22**), in minor amount, and GA_{39} (**28**), in major amount, were identified by GC/MS in extracts of seed of maturity index 45. An authentic sample of GA_{39} (**28**) was obtained as described in the following paper [4].

In the third series of experiments the constituents of dry, mature seeds were investigated by GC/MS after TLC of the acidic ethyl acetate-soluble acids and of the *n*-

Table 1.

Compounds identified	Source (maturity index) and relative abundance of compounds*												
	39%		39% + cellulase	61%		61% + cellulase		74%		74% + cellulase		100%	100% + cellulase
	Endo-sperm	Embryo	Endo-sperm	Endo-sperm	Embryo	Endo-sperm	Embryo	Endo-sperm	Embryo	Endo-sperm	Embryo	Embryo	Embryo
c, *t*-ABA (1)								x			x	xxx	xx
t, *t*-ABA (2)								x			x	x	x
Phaseic acid (3)						xx	xx			xxx	xx	x	xx
GA_{12} (4)				x				xx		x			
GA_{13} (5)		xxxxx		xx	xxxxx	xx	xxx	xx				xxxx	
GA_{25} (6)		xx			xx		x						
GA_{43} (7)	xxxx	xx	xxxx	xxxx	xx	xxxx		xxx		xxx		xx	
GA_{49} (9)	xx	x	xx	xx		xxx	x	xxx		xx	x	x	x
13-Hydroxykaurenoic acid (10)						xx							
7β,13-Dihydroxykaurenoic acid (11)				x				xx					
6β,7β-Dihydroxykaurenoic acid (12)	xx					x		xx					
6β,7β,13-Trihydroxykaurenoic acid (13)	xx		xx	xx		xx	x	xx	xx			x	
7β,16ξ,17-Trihydroxykauranoic acid (14)						xx							
6β,7β,16ξ,17-Tetrahydroxykauranoic acid (15)						x							
6,7-Secokauren-6,7,19-trioic acid or 6,7-seco-7-oxokauren-6,19-dioic acid (16)				x	xxx	xx	xx	x	xx	xx	xx	xxx	
7β,12α-Dihydroxykaurenolide (17)	xxxx	xx	xx	xxx	xxx	xx	xx	xxxx	xx	xx	x	xx	
12α-HydroxyGA_{12} (18)			xx			x						x	
12α-HydroxyGA_{14} (19)	xxxx		xx	xxx	xxx	xx	xx	xxx		xx		xx	
12α-HydroxyGA_{25} (20)					xxx		xx						
12α-HydroxyGA_4 (GA_{58}) (21)	xxxx		xxxxx	xxx		xxxx	xxx	xx		xxxx	xxx	xxxx	
12α-HydroxyGA_{37} (23)	xx			xxx				xxx					
7β,12β-Dihydroxykaurenoic acid (24)				xxx		xxx		xxx					x
6β,7β,12β-Trihydroxykaurenoic acid (25)	xxxxx	xxxx	xxxx	xxxxx	xxxxx	xxxx	xxxxx	xxxx	xxxx	xxxxx	xxxx	xxxx	xxxx
6,7-Seco-16ξ,17-dihydroxy-6,7-dioxokauranoic acid (26)			xxxxx			xxxxx	xxx			xxxxx	xxxxx		xx
3-IsopentanoylGA_{39} (27)		xx		xxx			xx		xxx		xxx	xxx	xx

*Relative TIC intensities within each GC/MS run.

butanol-soluble acids, hydrolysed with cellulase. The same compounds as before were detected in the hydrolysed *n*-butanol fraction with the addition of IAA, GA_{48} (**8**) and several unidentified kaurenoids. However, in the ethyl acetate-soluble acids, only GA_{39} and GA_{43} were present and possibly 12α-hydroxyGA_{25} (**20**) and GA_{48} (**8**) or GA_{49} (**9**).

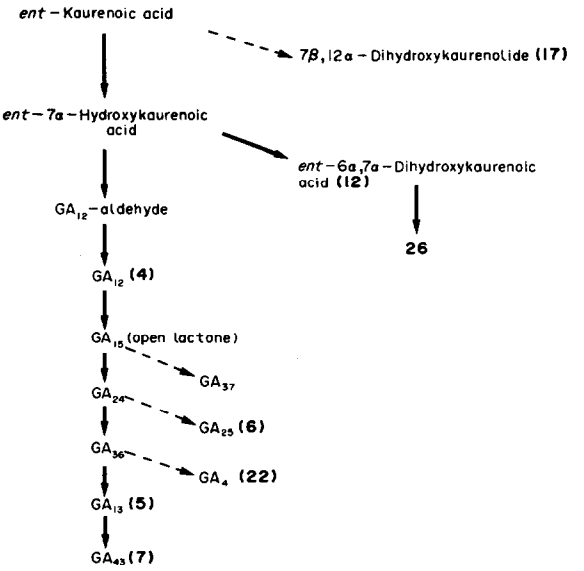
DISCUSSION

The occurrence of 12-hydroxylated GAs and *ent*-kaurenoids in seed of *C. maxima* is not unexpected since 7β,12α-dihydroxykaurenolide (**17**), GA_{39} (**28**), GA_{48} (**8**) and GA_{49} (**9**) have been previously isolated from immature seeds of *C. pepo* by Fukui *et al.* [7]. These authors assigned the 12α-hydroxy stereochemistry to the kaurenolide (**17**) on the basis of the following NMR evidence. The 12-proton triplet (J = 8 Hz) was similar to that in *ent*-12β,17-dihydroxy-16βH-kauranoic acid (**29**) [8] and different from that in the *ent*-12α-hydroxy-isomer (**30**). Fukui *et al.* [7] also reported the chemical shifts of the 17-protons in the kaurenolide (**17**) were not deshielded on changing the solvent from $CDCl_3$ to C_5D_5N which is consistent with a 12α-hydroxyl [9, 10]. However they did not record the chemical shift data for the kaurenolide (**17**) in C_5D_5N. We therefore re-examined the ^1H NMR of the kaurenolide from *C. pepo* in $CDCl_3$ and C_5D_5N solutions, finding the data (see Experimental) to be inconclusive. However, as shown in the following paper [4] the Kovats retention indices and co-injection show conclusively that the kaurenolide from *C. maxima* and *C. pepo* is the 12α-hydroxy isomer (**17**).

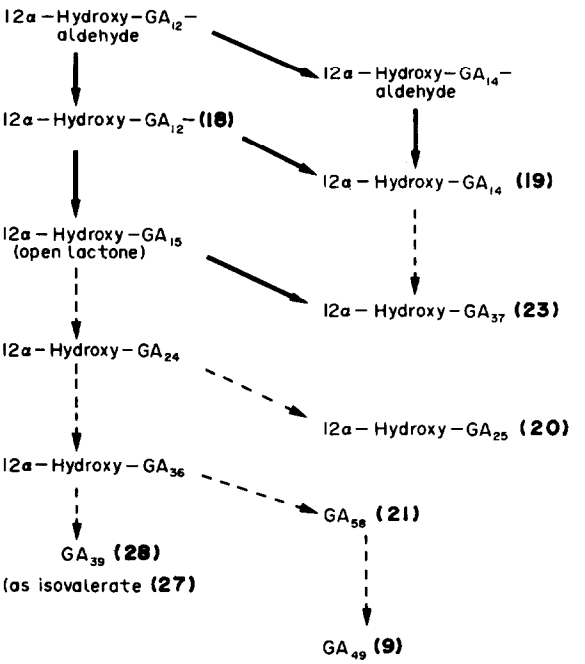
The stereochemistry of the 12-hydroxyl in the new compounds, described in the present study, was determined as described in the accompanying papers [4, 5]. Since 12α-hydroxyGA_4 (**21**) has been isolated [5] it has been allocated [11] the GA number, GA_{58}. The allocation of GA numbers to the other native 12α-hydroxyGAs, reported in this paper, must await their isolation and full chemical characterization.

Scheme 1 shows the biosynthetic pathway of non- and 3β-hydroxylated GAs as obtained in the cell-free system from *C. maxima* endosperm [1–3]. A comparison of the scheme with the endogenous compounds listed in Table 1 shows good agreement. Thus 7β,12α-dihydroxykaurenolide (**17**), compound **26** and GA_{43} (**7**) are major constituents of the endosperm and also major end products in the cell-free system. In the extracts of embryos, GA_{13} (**5**) takes the place of GA_{43} (**7**) as a major component, which indicates that the conversion of GA_{13} to GA_{43} is less effective in this tissue. GA_4 (**22**) and GA_{25} (**6**) are rarely found, minor constituents *in vivo* and also minor end products in the cell-free system. The only discrepancies found with respect to end products are GA_{37} (formula not shown here), which is a minor product in the cell-free system but not found in the extracts, and the diacid **16**, which has not yet been identified as a product of the cell-free system. Except for compound **26**, the compounds were found mainly or entirely in free form in the immature seed. Of the several intermediates obtained in the cell-free system, only *ent*-6α,7α-dihydroxykaurenoic acid (**12**) and GA_{12} (**4**) were sporadically found as minor constituents of the endosperm. This is not surprising, since intermediates usually do not accumulate *in vivo*.

Scheme 2 shows a hypothetical pathway of 12α-hydroxylated GAs in *C. maxima* based on new results

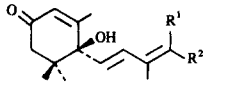


Scheme 1. GA biosynthesis in *C. maxima* cell-free system (cf. ref. [1]).

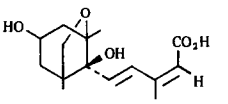


Scheme 2. Hypothetical scheme of biosynthetic pathway of 12α-hydroxylated GAs. Reactions represented by full arrows have been shown to occur in the *C. maxima* cell-free system [6].

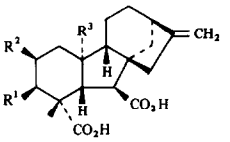
obtained with the cell-free system [6] and by analogy with Scheme 1. Table 1 shows that 12α-hydroxyGA_{14} (**19**), GA_{39} (**27**, **28**) and GA_{58} (**21**) are major endogenous components. 12α-HydroxyGA_{37} (**23**) and GA_{49} (**9**) are present in substantial amounts whereas 12α-hydroxyGA_{25} (**20**) was found in a few cases only. Like GA_{25} (**6**) and GA_{13} (**5**), the corresponding 12α-derivatives



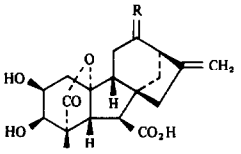
1 $R^1 = CO_2H, R^2 = H$
2 $R^1 = H, R^2 = CO_2H$



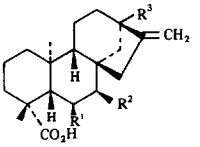
3



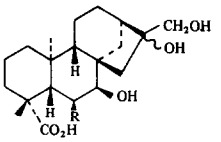
4 $R^1 = R^2 = H, R^3 = Me$
5 $R^1 = OH, R^2 = H, R^3 = CO_2H$
6 $R^1 = R^2 = H, R^3 = CO_2H$
7 $R^1 = R^2 = OH, R^3 = CO_2H$



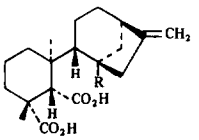
8 $R = H, \beta-OH$
9 $R = H, \alpha-OH$



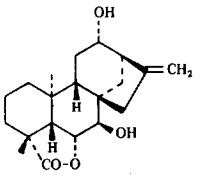
10 $R^1 = R^2 = H, R^3 = OH$
11 $R^1 = H, R^2 = R^3 = OH$
12 $R^1 = R^2 = OH, R^3 = H$
13 $R^1 = R^2 = R^3 = OH$



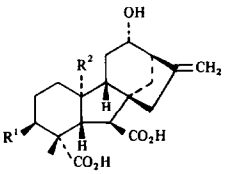
R
14 H
15 OH



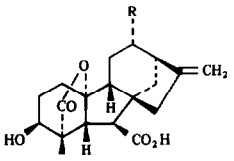
16 $R = CO_2H$ or CHO



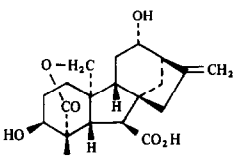
17



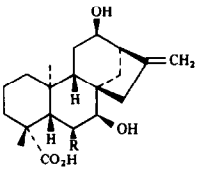
18 $R^1 = H, R^2 = Me$
19 $R^1 = OH, R^2 = Me$
20 $R^1 = H, R^2 = CO_2H$



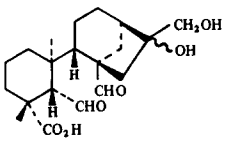
R
21 OH
22 H



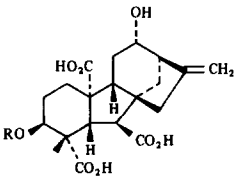
23



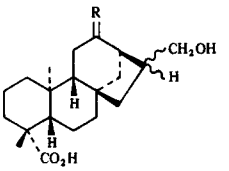
R
24 H
25 OH



26



27 $R = Me_2CHCH_2CO$
28 R = H



29 $R = H, \alpha-OH$
30 $R = H, \beta-OH$

are found mainly in the embryos. 12α-HydroxyGA_{37} (**23**) is found in the endosperm only, whereas 12α-hydroxyGA_{12} (**18**) and -GA_{14} (**19**) are found in both endosperm and embryos. The accumulation of 12α-hydroxyGA_{14} makes it less probable that this compound is an intermediate in the formation of GA_{58} (**21**) and GA_{49} (**9**), the two main C_{19}-GAs of the seeds. The latter are found in both endosperm and embryos but have not yet been obtained in the cell-free system from GA precursors [6]. GA_{58} and GA_{49} were partly present in bound or conjugated form in the embryos, GA_{39} (**28**) was conjugated with iso-valerate in most cases, but all other components were found free in the immature seeds. In contrast, most components of the dry, mature seeds had to

be released with cellulase before they could be extracted with ethyl acetate.

Three groups of kaurenoic acid derivatives with deviating hydroxylation patterns were found in the seeds. Compounds **24** and **25** are major constituents, curiously showing 12β-hydroxylation. Whereas **24** was found in the endosperm only, **25** was also present in the embryos. The second group consists of the 13-hydroxylated compounds **11**, **12** and **13**, which are minor components found mainly in the endosperm.Limited microsomal 13-hydroxylation has also been observed in the cell-free system under special conditions [6]. The third group consists of the very minor 16,17-hydroxylated compounds **14** and **15**, which are found in the endosperm only. Since neither GA_{12}-aldehyde nor GAs with 12β-, 13- and 16,17-hydroxylations were found, it is likely that the compounds **11**, **14** and **24** do not undergo ring contraction and thus do not serve as GA precursors.

EXPERIMENTAL

Extraction 1 (*Table* 1). (a) Plant material. *Cucurbita maxima* L cv 'Gelbe Riesenmelone mit Vernetzter Haut' was grown in the field. Endosperm and embryos were removed separately from four fruits of different maturity. The endosperm was homogenized lightly, the cotyledons were washed with K-Pi buffer (0.5 M, pH 8.0) to remove traces of endosperm and homogenized in the same buffer (2 ml per g). The following yields were obtained:

Maturity index (%)	Endosperm (ml)	Embryo (g)
39 ± 2	47	12
61 ± 2	25	15
74 ± 1	20	27
100 ± 0	0	78

The maturity index is the length of the cotyledons expressed as a percentage of the length of the seed lumen in a sample of 10 seeds [12]. Standard errors of the means are given.

(b) Extraction of unconjugated compounds. One half of each homogenate (endosperm or embryo) was used. The pH was adjusted to 3.0, Me_2CO (1:1) was added and lipid-soluble material was extracted (× 3) with EtOAc (1:2). The organic phase was washed with H_2O (10 ml) and taken to dryness *in vacuo*. The residue was dissolved in K-Pi buffer (0.5 M, pH 8.0, 1 ml) and petrol (40–60°, 1 ml). The petrol phase was discarded and the aq. phase was washed once more with petrol (1 ml). The pH of the aq. phase was adjusted to 3.0, Me_2CO (1 ml) was added and acidic lipid soluble material was extracted (× 3) with EtOAc (1 ml).

(c) Extraction after cellulase treatment. The other half of each homogenate was treated with cellulase before extraction. The pH of the homogenate was adjusted to 4.5 with HCl. Cellulase (Practical grade from *Aspergillus niger* and Serva, Heidelberg, 20 mg per ml of homogenate) was added and the mixture was incubated at 37° for 16 hr. The pH was then adjusted to 3.0 and the acidic fraction was extracted as above. By this procedure both originally free acids and originally conjugated acids are obtained in this fraction.

The compounds, identified by GC/MS, are listed in Table 1.

Extraction 2. In a second series of extractions seeds with maturity indices of 45% and 40% were harvested. Endosperm (10 ml from each) and cotyledons were stored at −20° after they had been removed from the seeds. The frozen endosperm was stirred with MeOH (80% 3:1) at 2° for 60 min. The frozen cotyledons were ground in a blender with MeOH (80% 3:1) and solid CO_2. In both cases the solids were separated by centrifugation. The pellets were extracted twice more with 80% MeOH. The combined extracts from endosperm or cotyledons were concd *in vacuo*, the pH of the resulting aq. soln was adjusted to 2.5 and material was extracted (× 4) with EtOAc (1:2). The organic phase was washed with a small vol. of H_2O. Acidic compounds were extracted (× 3) with K-Pi (0.2 M, 5 ml). The buffer soln was concd to 10 ml *in vacuo* and applied to a column of PVP (200 × 15 mm). GAs and related compounds were eluted with K_2HPO_4 (0.2 M). The first 30 ml were discarded. The pH of the next 200 ml was adjusted to 3.0 and acidic compounds were extracted (× 4) with EtOAc (50 ml). The EtOAc extract was washed with a small vol. of H_2O and taken to dryness *in vacuo*. The following compounds were identified by GC/MS of the derivatized extracts: endosperm (45%)—**5**, **7**, **9**, **13**, **18**, **19**, **22** and **23**; cotyledons (45%)—**5**, **6**, **9**, **13**, **20**, **26**, **27** and **28**; endosperm (40%)—**5**, **7**, **9**, **13**, **19**, **22** and **23**; cotyledons (40%)—**7** and **26**.

Extraction 3. One hundred dry, mature seeds of *C. maxima* were peeled and homogenized with 50% MeOH (5:1, v/w). After stirring for 2 hr at 2° in the dark, the mixture was centrifuged at 5000 *g* for 5 min. The pellet was extracted twice more in the same manner and the supernatants were combined. The solvent was reduced *in vacuo* to the watery residue which was extracted with EtOAc at pH 2.8. Organic acids were extracted from the EtOAc extract into K-Pi (0.1 M, pH 8.3) and, after the pH had been lowered to 2.8, re-extracted into EtOAc.

The acidic water phase after the first extraction with EtOAc was extracted (× 4) with BuOH. The extract was evaporated to dryness at 40° and completely dissolved in 20 ml H_2O. Cellulase from *A. niger* (10 mg/g seed wt) and 3–4 drops of toluene were added. The pH was adjusted to 4.5 and the mixture was incubated with shaking for 24 hr at 31°. After incubation, pH was adjusted to 2.8 and GAs were extracted as described above. By this procedure only the originally conjugated acids were obtained in this fraction.

The following compounds were identified by GC/MS of the derivatized extracts: free components—**7**, **9** or **8**, **20**, **27** and **28**; components extracted after treatment of the BuOH-extract with cellulase—**8**, **9**, **17**, **18**, **19**, **20**, **21** , **23–25**, **18** and IAA.

GC/MS identification. The EtOAc-soluble acids were methylated (CH_2N_2) and trimethylsilylated [$CF_3C(OSiMe_3)$=NMe]. The MeTMSi derivatives were analysed using a GEC-AEI MS30 and either a 2% QF-1 or 2% SE-33 packed column under the conditions described in ref. [13]. Some of the extracts were subsequently re-analysed using a VG 7050 mass spectrometer and an OV-1 WCOT fused silica column (25 m × 0.2 mm i.d.) with He carrier gas at 2 bar, temp. programmed from 60° to 150° at 13°/min, then to 280° at 3°/min. The injector and interface temp. was 250° and the ionization voltage was 25 eV; a mixture of C_{22} to C_{32} *n*-alkanes were co-injected and the C-numbers determined. The derivatized extracts from mature seeds were analysed using a Finnigan GC/MS/DS Model 4015 and an open tubular SP2100 glass column (30 m × 0.24 mm i.d.).

Compounds **1–7**, **9–17**, **22** and **29** were identified by comparison with authentic samples.

GC/MS data for compounds **18–21**, **23–25** and **28** are recorded in the accompanying papers [2–4].

GC/MS data for the MeTMSi derivatives of the tentatively identified compounds are as follows: (a) *12α-Hydroxy* GA_{25} (**20**): *m/z* 492 $[M]^+$ (< 1%), 477 (20), 460 (54), 432 (30), 401 (31), 400 (73), 386 (18), 372 (77), 370 (64), 342 (14), 314 (13), 311 (47), 310 (97), 283 (97), 282 (100), 267 (11), 256 (14), 251 (18), 242 (14), 223 (65), 207 (93), 197 (23). (b) *Compound* **26**: *m/z* 524 $[M]^+$ (1%), 509 (4), 421 (100), 403 (65), 389 (86), 327 (45), 237 (50), 197 (23), 165 (42), 147 (28), 137 (58), 109 (64), 75 (47), 73 (36). (c) GA_{39}-3-*isovalerate* (**27**): *m/z* 592 $[M]^+$ (0%), 577 $[M-15]^+$ (0.25), 560 (6), 500 (17), 430 (17), 410 (21), 398 (32), 370 (18), 368 (16), 340 (49), 308 (100), 281 (39), 280 (66), 249 (22), 221 (51), 75 (48). (d) GA_8:

m/z 594 $[M]^+$ (100%), 504 (12), 448 (24), 397 (13), 379 (11), 376 (12), 375 (13), 311 (10), 238 (19), 221 (11), 207 (25), 194 (18), 193 (11), 129 (16). (e) *GA_{58}-1(10),16-dienoic acid: m/z* 520 $[M]^+$ (0%), 515 $[M-15]^+$ (4.5), 430 (21), 398 (34), 370 (61), 340 (21), 311 (33), 308 (41), 282 (24), 281 (51), 280 (75), 254 (51), 221 (100), 195 (22), 193 (21), 179 (21), 129 (21). (f) *2β-HydroxyGA_{39} 20,12-lactone: m/z* 564 $[M]^+$ (2%), 549 (1), 489 (7), 474 (6), 448 (9), 415 (100), 243 (19), 173 (10).

12α-Hydroxykaurenolide (**17**). ^{1}H NMR ($CDCl_3$): δ0.88 (*s*, H_3-20), 1.28 (*s*, H_3-18), 1.73 (*d*, *J* = 6.5 Hz, H-5), 3.80 (*t*, *J* = 8.0 Hz, H-12), 4.41 (*d*, *J* = 6.5 Hz, H-7), 4.61 (*t*, *J* = 6.5 Hz, H-6), 5.01 and 5.13 (both *s* (*br*), 17-H_2); δ(C_5D_5N) 0.96 (*s*, H_3-20), 1.30 (*s*, H_3-18), 1.91 (*d*, *J* = 6.5 Hz, H-5), 4.19 (*t*, *J* = 8.0 Hz), 4.79 (*br*, H-7), 5.10 (*t*, *J* = *ca* 6.0 Hz, H-6), 5.18 and 5.23 (both *s* (*br*), H_2-17).

GC data for MeTMSi derivatives of gibberellins A_{48} (**8**) *and A_{49}* (**9**). Under the conditions described for capillary GC/MS: GA_{48} MeTMSi, R_t 32 min 55 sec, Kovats ret. index 2787; GA_{49} MeTMSi, R_t 33 min 51 sec, Kovats ret. index 2831.

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