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GIBBERELLINS IN SEEDS OF *HELIANTHUS ANNUUS*

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IN MEMORY OF TONY SWAIN, 1922–1987

Key Word Index—*Helianthus annuus*; Compositae; sunflower; native gibberellins; new 15β-hydroxygibberellins; microbiological conversion of *ent*-15α-hydroxykaur-16-en-19-oic acid; *Gibberella fujikuroi*; mutant B1-41a.

Abstract—Fourteen gibberellins (GAs) have been identified in seeds of *Helianthus annuus*. They comprise the previously known GA_1, GA_4, GA_{19}, GA_{20} and GA_{45} and nine new 15β-hydroxyGAs. Of the new GAs, 15β-hydroxyGA_{20} and 15β-hydroxyGA_1 have been previously synthesized chemically from GA_3 and are now assigned the numbers GA_{67} and GA_{72}. The structures of 15β-hydroxyGA_{15}, 15β-hydroxyGA_{24} and 15β-hydroxyGA_{25} were established by their formation from the incubation of *ent*-15α-hydroxykaur-16-en-19-oic acid with cultures of *Gibberella fujikuroi*, mutant B1-41a; these new GAs have been respectively assigned the numbers, GA_{64}, GA_{65} and GA_{66}. The remaining four GAs have been tentatively assigned the structures, 15β-hydroxyGA_{17}, 15β-hydroxyGA_{19}, 15β-hydroxyGA_{44} and 15β-hydroxyGA_{53} on the basis of the mass spectra of their MeTMSi derivatives.

Abscisic acid, *trans*-abscisic acid, dihydrophaseic acid, dioxindole-3-acetic acid, *ent*-7α,16α,17-trihydroxy-16αH-kauranoic acid and *ent*-7α,16β,17-trihydroxy-and *ent*-6α,7α,16β,17-tetrahydroxy-16βH-kauranoic acids have also been detected in seeds of *Helianthus annuus* by capillary GC-MS. From the incubation of *ent*-15α-hydroxykaur-16-en-19-oic acid with *Gibberella fujikuroi*, mutant B1-41a, *ent*-7α,15α-dihydroxykaur-16-en-19-oic acid and 7β,15β-dihydroxykaurenolide have been isolated.

INTRODUCTION

The flower heads of the sunflower, *Helianthus annuus* L., contain [1, 2] *ent*-kaur-16-en-19-oic acid (**1**) and *ent*-12,16-cyclokauranoic acid (trachylobanic acid) (**3**). Extracts have also been shown [3] to contain smaller amounts of *ent*-13-methyl-17-nor-8β,13β-kaur-15-en-19-oic acid (beyerenoic acid) (**5**) and an ester fraction which gave *ent*-15β-hydroxykaur-16-en-19-oic acid (**2**) and *ent*-15β-hydroxytrachylobanic acid (**4**) on hydrolysis. Since *ent*-kaurenoic acid (**1**) is a biogenetic precursor of gibberellins (GAs), and since seeds are a relatively rich source of GAs in other species [4], we have investigated the native GAs in seeds of *H. annuus*.

RESULTS

Seeds of *H. annuus* were collected at three stages of maturity—mature, almost mature and immature. Each group was extracted with aqueous methanol and the extract was partitioned to give an ethyl acetate-soluble acidic fraction. The acidic fractions from each group of seeds were subjected to RP-HPLC and the HPLC fractions were bioassayed using the Tan-ginbozu dwarf rice assay [5]. Adjacent bio-active and bio-inactive fractions from the HPLC were combined and, after methylation and trimethylsilylation, were analysed by capillary GC/MS. The identified compounds are shown in Table 1.

The known compounds, dioxindole-3-acetic acid, abscisic acid, *trans*-abscisic acid, dihydrophaseic acid, GA_1 (**6**), GA_4 (**7**), GA_{19} (**9**) and GA_{20} (**8**) were identified by direct comparison of the mass spectra and Kovats Retention Indices (K_I) of their MeTMSi derivatives with reference data of standards. The structures of the new GAs were determined as described in the following sections.

Assignment of structures to GA_{64} (**13**), GA_{65} (**15**) and GA_{66} (**17**)

The mass spectra of the MeTMSi derivatives of each of these GAs contained an ion at *m/z* 156, typical of 15-hydroxyGAs and of *ent*-15-hydroxykaurenoids [6, 7]. With this information, *ent*-15α-hydroxykaur-15-en-19-oic acid (**19**) was incubated with the fungus, *Gibberella fujikuroi*, mutant B1-41a, which is blocked [8] for GA-biosynthesis at the step before *ent*-kaurenoic acid (**1**) and which converts analogues of *ent*-kaurenoic acid into the corresponding analogues of the fungal GAs [9, 10]. Among the products were three compounds which could be assigned the structures, 15β-hydroxyGA_{15} (**13**), 15β-hydroxyGA_{24} (**15**) and 15β-hydroxyGA_{25} (**17**) from their mass spectra and from their formation from *ent*-15α-hydroxykaurenoic acid (**19**). 15β-HydroxyGA_{15} (**13**), 15β-hydroxyGA_{24} (**15**) and 15β-hydroxyGA_{25} (**17**) have previously been isolated and characterised [11] as metabolites of *ent*-15α-hydroxykaurenoic acid (**19**) in cultures of a wild-type strain of *G. fujikuroi* in the presence of the inhibitor, 1-*n*-decylimidazole, of GA-biosynthesis. Since they are now shown to occur naturally, they have been assigned [12] the numbers GA_{64} (**13**), GA_{65} (**15**) and GA_{66} (**17**).

Table 1. Compounds identified in seeds of *Helianthus annuus* by full scan GC-MS

Compound	K_I	Mature	Almost mature	Immature
GA_1 (**6**)	2663	—	—	+
GA_4 (**7**)	2494	—	+	—
GA_{19} (**9**)	2591	—	—	+
GA_{20} (**8**)	2476	—	+	+
GA_{45} (**10**)	2478	—	+	—
GA_{64} (**13**)	2729	—	+	—
GA_{65} (**15**)	2561	—	+	—
GA_{66} (**17**)	2545	—	+	+
GA_{67} (**22**)	2613	+	+	+
GA_{72} (**23**)	2822	+	—	+
15β-HydroxyGA_{17} (**18**)	2636	+	+	+
15β-HydroxyGA_{19} (**16**)	2671	+	+	+
15β-HydroxyGA_{44} (**14**)	2855	+	+	—
15β-HydroxyGA_{53} (**24**)	2568	—	+	—
Abscisic acid	2026	—	+	+
t-Abscisic acid	2103	—	+	+
Dihydrophaseic acid	2150	+	—	—
Dioxindole-3-acetic acid	1872	+	+	+
ent-7α,16α,17-Trihydroxy-16α-H-kauranoic acid	2846	—	+	—
ent-7α,16β,17-Trihydroxy-16β-H-kaurenoic acid	2863	—	+	—
ent-6α,7α,16α,17-Tetrahydroxy-16α-H-kauranoic acid	2956	—	+	—

From the incubation of *ent*-15α-hydroxykaurenoic acid (**19**) with cultures of *G. fujikuroi*, mutant B1-41a, two new *ent*-kaurenoids were isolated and shown to be *ent*-7α,15α-dihydroxykaurenoic acid (**20**) and 7β-15β-dihydroxykaurenolide (**21**) by elemental analyses, ^{1}H NMR and mass spectrometry.

Identification of the 13,15β-dihydroxy gibberellins

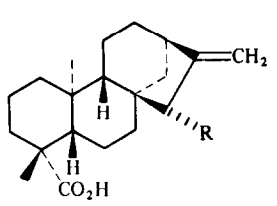
The mass spectra of the MeTMSi derivatives of these compounds contained ions at *m/z* 229 (*m/z* 244 − Me), at *m/z* 244 which corresponds to the *m/z* 156 ion (characteristic [6, 7] of 15β-hydroxyGAs) plus an additional OTMSi substituent and at *m/z* 295/6 which correspond to the ions *m/z* 207/8 (characteristic of a 13-hydroxyGA), plus an additional OTMSi substituent. The ions at *m/z* 229, 244 and 295/6 are therefore indicative of 13,15β-dihydroxyGAs. On this basis, and by analogy with the structures deduced for GA_{64} (**13**), GA_{65} (**15**) and GA_{66} (**17**) in the preceding section, the structures **14**, **16**, **18**, **22**, **23** and **24** were tentatively deduced for the six new 13,15β-dihydroxyGAs. In the meantime structures **22** and **23** have been confirmed by partial synthesis from GA_3 (**25**) by Dolan and MacMillan [13] and assigned [12] the GA numbers, GA_{67} and GA_{72}, respectively.

The structures for the other four 13,15β-dihydroxyGAs, namely 15β-hydroxyGA_{17} (**18**), 15β-hydroxyGA_{19} (**16**), 15β-hydroxyGA_{44} (**14**) and 15β-hydroxyGA_{53} (**24**) remain tentative. Unsuccessful attempts were made to confirm these structures by preparing them from the incubation of *ent*-13,15α-dihydroxykaurenoic acid (**26**) in cultures of *G. fujikuroi*, mutant B1-41a. Under a variety of conditions no GAs were formed and unidentified tri- and tetrahydroxykaurenoic acids were the only metabolites detected by capillary GC/MS. Similar results were obtained with the 15α-epimer (**28**) and no metabolites were detected from the 13-acetate (**27**). These results were unexpected since cultures of *G. fujikuroi* have been shown to convert *ent*-15α-hydroxykaurenoic acid (**19**), *ent*-13-hydroxykaurenoic acid (**29**), *ent*-13-acetoxykaurenoic acid (**30**) and *ent*-13-methyl-17-nor-16-oxo-8β,13β-kauranoic acid (**35**) into the corresponding analogues of the normal fungal GAs [6, 9, 10].

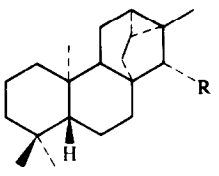
Preparation of substrates for fungal transformations

The 13,15-dihydroxylated derivatives of *ent*-kaurenoic acid, used for the incubations with *G. fujikuroi*, mutant B1-41a, are new compounds. They were prepared from *ent*-13-hydroxykaurenoic acid (**29**) via the 13-acetate (**30**) which was 15α-hydroxylated by treatment with selenium dioxide and *t*-butylhydroperoxide. The resultant 15α-alcohol (**28**) was oxidised with Jones reagent to the 15-ketone (**31**) which, on reduction with sodium borohydride in methanol gave a mixture of the 15β- and 15α-alcohols **27** and **28** in a 3:2 ratio. Separation of the mixture, then deacetylation of **27** gave the required *ent*-13,15α-dihydroxykaurenoic acid (**26**). The 13-acetates were used because reduction of the 13-hydroxy-15-one (**32**), obtained from *ent*-13-hydroxykaurenoic acid (**29**) via the 15α-alcohol (**33**) gave only the 15α-alcohol (**33**), and none of the required 15β-alcohol (**26**), under a range of conditions. The influence of the remote 13-function on the stereochemical outcome of reduction of the 15-one is noteworthy.

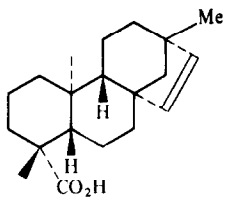
ent-15α-Hydroxy- and 13,15α-dihydroxykaurenoic acids (**19** and **26**) were respectively isomerised by mineral acids to the 15-ketones **36** and **37**, presumably by 15,16-



1 R = H
2 R = OH



3 R = H
4 R = OH



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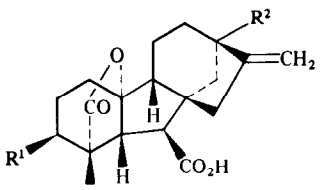
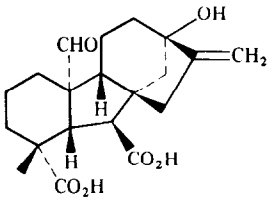


6 R^1 = R^2 = OH(GA_1)
7 R^1 = OH, R^2 = H(GA_4)
8 R^1 = H, R^2 = OH(GA_{20})



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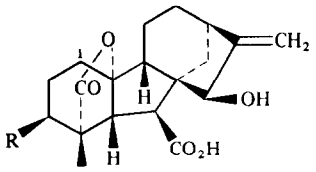
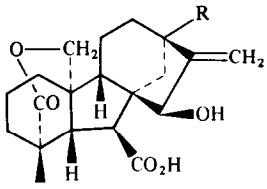
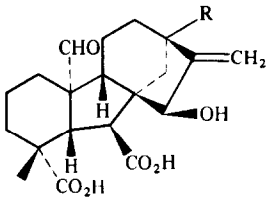


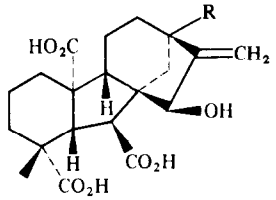
10 R = H(GA_{45})
11 R = OH(GA_{63})
12 R = OH, 1-ene(GA_{68})



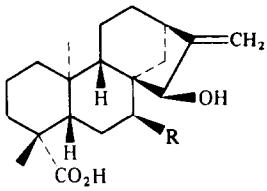
13 R = H(GA_{64})
14 R = OH



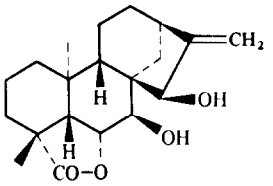
15 R = H(GA_{65})
16 R = OH



17 R = H(GA_{66})
18 R = OH



19 R = H
20 R = OH



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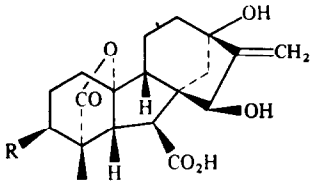


22 R = H(GA_{67})
23 R = OH(GA_{72})

hydride shifts as previously shown [14] for *ent*-15α-hydroxykaur-16-ene. The corresponding ent-15β-alcohols (**33** and **34**) were stable to acid.

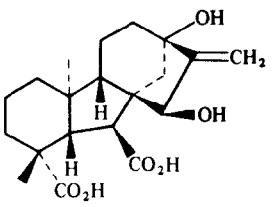
DISCUSSION

Previously only three 15β-hydroxyGAs, GA_{45} (**10**), GA_{63} (**11**) and GA_{68} (**12**), were known to occur naturally [6, 13, 15, 16]. Of the ten 15β-hydroxyGAs now identified in seeds of *H. annuus*, three are C_{19}-GAs of which two, GA_{67} (**22**) and GA_{72} (**23**), are 13-hydroxylated and one, GA_{45} (**10**), is not. The others are C_{20}-GAs of which three, GA_{64} (**13**), GA_{65} (**15**) and GA_{66} (**17**), are not 13-hydroxylated and four with the tentative structures 15β-hydroxyGA_{17} (**18**), 15β-hydroxyGA_{19} (**16**), 15β-hydroxyGA_{44} (**14**) and 15β-hydroxyGA_{53} (**24**) are 13-hydroxylated. The occurrence of these 15β-hydroxy-C_{20}-GAs suggests that 15β-hydroxylation occurs early in parallel 13-hydroxylation and non-13-hydroxylation pathways from GA_{12}-aldehyde. It is unlikely that 15β-hydroxylation occurs before GA_{12}-aldehyde since no *ent*-15α-hydroxykaurenoids have been found in *H. annuus*. Indeed only *ent*-15β-hydroxykaurenoic acid has been found [2].

Whether the 13,15β-dihydroxy- and 15β-hydroxy-C_{20}-GAs represent convergent pathways to GA_{67} (**22**) and GA_{72} (**23**), and whether GA_{20} (**8**) is 15β-hydroxylated to GA_{67} (**22**) which is then 3β-hydroxylated to GA_{72} (**18**) require to be tested experimentally.

EXPERIMENTAL

Plant material. Seeds of *H. annuus* were collected at three stages of maturity: (*a*) fully mature seeds (129 g); (*b*) almost mature seeds (41 g) which bore black stripes but were still soft and (*c*) immature seeds (265 g) which were soft, white and



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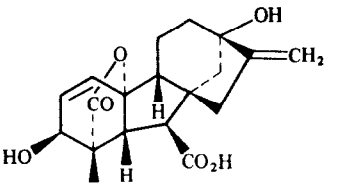

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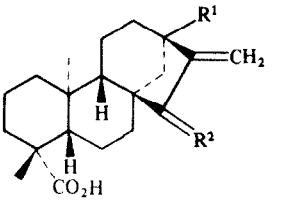
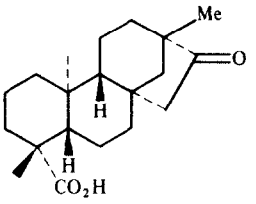


26 R^1 = OH, R^2 = H, β-OH
27 R^1 = OAc, R^2 = H, β-OH
28 R^1 = OAc, R^2 = H, α-OH
29 R^1 = OH, R^2 = H_2
30 R^1 = OAc, R^2 = H_2
31 R^1 = OAc, R^2 = O
32 R^1 = OH, R^2 = O
33 R^1 = OH, R^2 = H, α-OH
34 R^1 = H, R^2 = H, α-OH



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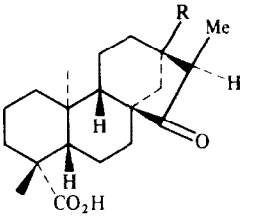
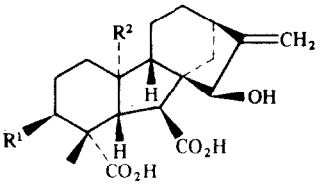


36 R = H
37 R = OH



38 R^1 = H, R^2 = Me
39 R^1 = OH, R^2 = Me
40 R^1 = OH, R^2 = CO_2H

covered in an oily layer which was removed prior to extraction by washing with EtOAc.

Seed extraction. All glassware used was cleaned with chromic acid. Each type of seed was macerated in MeOH–H_2O (4:1, 500 ml) using a Waring blender. After 2 days, the mixture was filtered and the residue was re-extracted with MeOH–H_2O (4:1, 500 ml) for 1 day. The combined filtrates were evapd to dryness *in vacuo* at 40°, toluene being added to asssist in the removal of the H_2O.

The residual oil in MeOH–H_2O (4:1, 100 ml) was washed with petrol (bp 60–80°, 3 × 50 ml) then evapd to dryness as before. After partitioning between water (pH 3.0, 100 ml) and EtOAc (3 × 100 ml), the organic layers were washed with H_2O (20 ml) and evapd to dryness under vacuum. Preliminary GC/MS of a portion of the residue as the MeTMSi derivative indicated a complex mixture. The residue was then partitioned between H_2O (pH 8.0, 25 ml) and EtOAc (3 × 25 ml). The combined EtOAc fractions were washed with H_2O (5 ml) and the combined aq. layers were adjusted to pH 2.0 with 2M aq. HCl then extracted with EtOAc (3 × 30 ml). The organic layers were washed with H_2O (10 ml) then evapd to give the EtOAc-soluble acids.

Purification and GC/MS analysis of the EtOAc-soluble acids. The crude fraction was dissolved in MeOH–H_2O (7:2, 2 ml), washed with petrol (bp 60–80°, 3 × 2 ml) and passed through a micropore filter. The filtrate was fractionated by HPLC using a C_{18}-Spherisorb ODS column (250 × 10 mm) and a linear gradient from MeOH–1% aq. AcOH (3:7) to 100% MeOH over 25 min at a flow-rate of 2.5 ml/min. Fractions were collected at 1 min intervals to provide a total of 30 fractions.

An aliquot (500 μl) from each fraction was evapd. Water (500 μl) and five 2 day old germinated seeds of *Oryza sativa* cv Tan-ginbozu were added to each. The seedlings were grown at 32° at 100% humidity under two 65/80 W warm white fluorescent tubes for 5 days. The lengths of the second leaf sheaths were measured. Adjacent bio-active and bio-inactive fractions were combined, dissolved in MeOH and treated with excess CH_2N_2 in Et_2O. The solutions were evapd and the residue was trimethylsilylated. The resultant MeTMSi derivatised fractions were analysed by capillary GC/MS; the MS and Kovats Retention Indices (K_I) were determined under the conditions previously described [17].

The HPLC fractions were examined, and the compounds identified, were as follows:

Mature seeds. Fractions 1–7: dioxindole-3-acetic acid, GA_{72} (**23**). Fractions 8–04: dioxindole-3-acetic acid, dihydrophaseic acid, GA_{67} (**22**), 15β-hydroxyGA_{44} (**14**), 15β-hydroxyGA_{19} (**16**), 15β-hydroxyGA_{17} (**18**). Fractions 15–23: no identifications.

Almost mature seeds. Fractions 1–7: not examined. Fractions 8–11: no identifications. Fractions 12–14: dioxindole-3-acetic acid, 15β-hydroxyGA_{44} (**14**), 15β-hydroxyGA_{19} (**16**), 15β-hydroxyGA_{17} (**18**). Fraction 15: abscisic acid, *trans*-abscisic acid, *ent*-7α,16α,17-trihydroxy-16αH-kaurenoic acid, *ent*-6α,7α,16α,17-tetrahydroxy-16αH-kaurenoic acid, 15β-hydroxyGA_{19} (**16**). Fractions 16–18: GA_{20} (**8**), GA_{45} (**10**), GA_{64} (**13**), GA_{65} (**15**), GA_{66} (**17**), 15β-hydroxyGA_{53} (**24**), *ent*-7α,16α,17-trihydroxy-16αH-kaurenoic acid, *ent*-7α,16β,17-trihydroxy-16βH-kaurenoic acid. Fractions 19–21: GA_4 (**7**), GA_{20} (**8**).

Immature seeds. Fractions 1–5 and 6–9: dioxindole-3-acetic acid. Fractions 10, 11: abscisic acid, *trans*-abscisic acid, GA_{19} (**9**), GA_{20} (**8**), GA_{66} (**17**). Fraction 12: no identifications. Fractions 13–19: GA_1 (**6**), GA_{67} (**22**), GA_{72} (**23**), 15β-hydroxyGA_{19} (**16**), 15β-hydroxyGA_{17} (**18**). Fractions 20–30: no identifications.

The K_I and MS data on which these identifications are based, are listed later together with the identified metabolites from the

incubation of *ent*-15α-hydroxykaur-16-en-19-oic acid (**19**) with cultures of *Gibberella fujikuroi*, mutant B1-41a.

Incubations of substrates in cultures of Gibberella fujikuroi, *mutant B1-41a.* (*a*) ent-15α-*Hydroxykaur-16-en-19-oic acid* (**19**). The acid (1 mg) was incubated for 5 days with resuspended mycelium of the B1-41a mutant and worked up as previously described [9]. The acid fraction in Me_2CO was methylated with ethereal CH_2N_2 and, after 15 min, the soln was evapd. The residue was extracted with CH_2Cl_2 (dried over CaH_2) to remove carbohydrates. The CH_2Cl_2 extract was evapd and the residue was trimethylsilylated at 60° for 5 min with trimethylsilyl chloride–hexamethyldisilazane–pyridine (2:3:2). The resulting MeTMSi derivatives were analysed by capillary GC/MS as described previously [17]. The following compounds were identified on the basis of the MS and K_I data listed later: 15β-hydroxyGA_{12} (**38**); 15β-hydroxyGA_{14} (**39**); 15β-hydroxyGA_{13} (**40**); GA_{45} (**10**); GA_{63} (**11**); GA_{64} (**13**); GA_{65} (**15**); and GA_{66} (**17**).

(*b*) ent-13,15α-*Dihydroxykaur-16-en-19-oic-acid* (**26**). The acid (1 mg) was added to each of two flasks containing resuspended mycelium. One was buffered to pH 3.0 and the other to pH 4.5. After 5 days incubation and the usual work-up, the products were examined by GC as the MeTMSi derivatives. Both cultures showed similar GC traces and the following compounds were detected by capillary GC/MS for the pH 4.5 culture: (i) unmetabolised substrate (**26**); unknown compound with K_I 2714 and *m/z* (rel. int.) 580 (M^+, 25), 490 (85), 475 (19), 462 (10), 431 (9), 400 (58), 392 (23), 362 (100), 244 (24), 229 (42), 194 (20), 191 (17); unknown compound with K_I 2726 and *m/z* (rel. int.) 668 (M^+, absent), 578 (7), 505 (21), 448 (24), 418 (13), 298 (13), 265 (25), 226 (28), 183 (16), 143 (40), 117 (25); unknown compound with K_I 2756 and *m/z* (rel. int.) 582 (3), 492 (34), 452 (91), 451 (69), 402 (32), 362 (87), 322 (46), 217 (33), 191 (52), 182 (23), 130 (28).

(*c*) ent-13,15β-*Dihydroxykaur-16-en-19-oic acid* (**33**). The acid (2 mg) was incubated as described in (*b*). The substrate was not detected in the products by capillary GC/MS but two unidentified compounds were observed (i) K_I 2558 and *m/z* (rel. int.) 580 (M^+, 100), 490 (68), 475 (34), 451 (71), 400 (58), 387 (31), 383 (55), 369 (77), 362 (54), 355 (46), 343 (19), 322 (21), 244 (19), 229 (36), 191 (30), 147 (13), 129 (6); (ii) K_I 2926 and *m/z* (rel. int.) 668 (M^+, 2), 578 (12), 448 (4), 398 (16), 381 (18), 368 (13), 357 (19), 355 (30), 269 (15), 209 (10), 191 (22), 147 (14), 75 (100).

(*d*) ent-13-*Acetoxy*-15α-*hydroxykaur-16-en-19-oic acid* (**27**). The substrate (1 mg) was incubated and worked-up as described in (*b*). After hydrolysis of the product (1 ml MeOH, 10 mg K_2CO_3, 18 hr) the only compound identified by capillary GC/MS was the MeTMSi derivative of *ent*-13,15α-dihydroxy kaurenoic acid (**26**).

Isolation of ent-7α,15α-*dihydroxykaur-16-en-19-oic acid* (**20**) *and* 7β,15β-*dihydroxykaurenolide* (**21**). The neutral extract from a large-scale incubation of *ent*-15α-hydroxykaurenoic acid (**19**) (400 mg) was separated by chromatography on column (300 × 25 mm) of silica gel (60–120 mesh, 50 g), eluted with EtOAc–petrol (bp 60–80°).

ent-7α,15α-*Dihydroxykaurenoic acid* (**20**). This compound was eluted with 40% EtOAc in petrol and crystallized from Me_2CO–petrol, mp 233–235°. (Found: C, 71.8; H, 9.3. $C_{20}H_{30}O_4$ requires C, 71.8; H, 9.0%). IR ν_{max}^{nujol} cm^{-1}: 3595, 3490, 1695 and 900; ^1H NMR (100 MHz, CD_3COCD_3): δ1.02 (3H, *s*, 20-H), 1.16 (3H, *s*, 18-H), 2.60 (1H, *br m*, 13-H, 3.68 (1H, *br s*, 15-H), 4.18 (1H, *br s*, 7-H), 4.91 and 5.06 (2H, each *br s*, 17-H); ^1H NMR (200 MHz, C_5D_5N): δ1.23 (3H, *s*, 20-H), 1.37 (3H, *s*, 18-H), 3.93 (1H, *s*, 15-H), 4.54 (1H, *s*, 7-H), 5.09 and 5.45 (2H, each *br s*, 17-H); EIMS (probe) 70 eV, *m/z* (rel. int.): 334 $[M]^+$ (5%), 319 (6), 317 (24), 316 (100),314 (48), 288 (65), 270 (48), 259 (46), 258 (64), 255 (49), 173 (41), 109 (62), 105 (56) and 104 (55).

The methyl ester crystallized from EtOAc, mp 142.5–144°; IR ν_{max}^{nujol} cm^{-1}: 3450, 3320 *br*, 1660 and 895; ^1H NMR (100 MHz, $CDCl_3$): δ0.85 (3H, *s*, 20-H) 1.15 (3H, *s*, 18-H), 2.66 (1H, *br s*, 13-H), 3.64 (3H, *s*, OMe), 3.70 (1H, *br s*, 15-H, 4.22 (1H, *br s*, 7-H), 4.93 and 5.07 (2H, each *br s*, 17-H).

ent-6β,7α,15α-*Trihydroxykaur-16-en-19-oic acid* 19,6-*lactone* (**21**). This compound was eluted with 50% EtOAc in petrol and was purified by prep. TLC with EtOAc–petrol–$MeCO_2H$ (30:50:2). It had R_f 0.20 crystallized from MeOH, mp 279–281°. (Found: C, 72.3, H, 8.5. $C_{20}H_{28}O_4$ requires C, 72.3; H, 9.0%). IR ν_{max}^{nujol} cm^{-1}: 3350 *br*, 1780, 1660 and 890; ^1H NMR (200 MHz, CD_3COCD_3): δ0.86 (3H, *s*, 20-H), 1.20 (3H, *s*, 18-H), 2.20 (1H, *d*, *J* = 6 Hz, 5-H), 2.56 (1H, *m*, 13-H), 3.67 (1H, *br t*, 15-H), 4.31 (1H, *d*, *J* = 6 Hz, 7-H), 4.75 (1H, *t*, *J* = 6 Hz, 6-H), and 5.0 (2H, *br s*, 17-H); EIMS (bis TMSi ether, probe) 70 eV, *m/z* (rel. int.): 476 $[M]^+$ (7), 461 (3), 433 (7), 387 (36), 386 (100), 156 (33) and 109 (17).

Synthesis of ent-13,15α-*dihydroxykaur-16-en-19-oic acid* (**26**). (*a*) *Enzymatic hydrolysis of stevioside to* ent-13-*hydroxykaur-16-en-19-oic acid* (**29**). Pectinol CG R-10 concentrate (Rohm and Haas) (50 mg), dissolved in KH_2PO_4 buffer (0.05 M, pH 4, 50 ml), was filtered into a soln of stevioside (5 g) in buffer (50 ml) and incubated for 24 hr at 50° with shaking. The pptd product was obtained by vacuum filtration, washed with H_2O and dried under vacuum. The crude *ent*-13-hydroxykaur-16-en-19-oic acid (**29**) (1.65 g) was crystallized from MeOH as needles, mp 204–206° (lit. ref. [10, 20] 199–200.5°, 208–210°). (Found: M^+ at *m/z* 318.2195. Calcd for $C_{20}H_{30}O_3$, 318.2195). IR ν_{max}^{nujol} cm^{-1}: 3480, 3300 *br*, 1695 and 890; ^1H NMR (100 MHz, $CDCl_3$): δ0.96 (3H, *s*, 20-H), 1.23 (3H, *s*, 18-H), 4.86 and 5.03 (2H, each *br s*, 17-H): EIMS (probe) 70 eV, *m/z* (rel. int.): 318 $[M]^+$ (100), 300 (38), 285 (20), 272 (10), 261 (10), 260 (26), 254 (14), 147 (10), 146 (11), 121 (83), 109 (18) and 43 (22).

(*b*) ent-13,15β-*Dihydroxykaur-16-en-19-oic acid* (**33**). The acid (**29**) (500 mg) in $CHCl_3$ (50 ml) was treated overnight with SeO_2 (80 mg) and *t*-BuO_2H (350 μl). MeOH (10 ml) was added, the soln filtered through Celite and evapd under a stream of N_2, *ent*-13,15β-dihydroxykaur-16-en-19-oic acid (**33**) (500 mg) crystallized from EtOH, mp 249–251° (Found: C, 72.4; H, 8.9%; M^+ at *m/z* 344.2137. $C_{20}H_{30}O_4$ requires C, 71.8; H, 9.0%; M^+, 334.2144). IR ν_{max}^{nujol} cm^{-1}: 3470, 3370 *br*, 1695 and 905; ^1H NMR (100 MHz, CD_3OD): δ0.97 (3H, *s*, 20-H), 1.20 (3H, *s*, 18-H), 3.75 (1H, *br s*, $W_{\frac{1}{2}}$ 4 Hz, 15-H), and 5.22 (2H, *br s*, 17-H); ^1H NMR (100 MHz, C_5D_5N): δ1.20 (3H, *s*, 20-H), 1.29 (3H, *s*, 18-H), 4.28 (1H, *br s*, 15-H), 5.66 and 5.74 (2H, each *s*, 17-H); EIMS (probe) 70 eV, *m/z* (rel. int.): 334 $[M]^+$ (72), 316 (60), 301 (24), 298 (17), 277 (19), 270 (26), 237 (49), 235 (38), 221 (34), 189 (22), 149 (42), 148 (42), 137 (100), and 121 (51).

(*c*) ent-13*Hydroxy*-15-*oxokaur*-16-*en*-19-*oic acid* (**32**). *ent*-13,15β-Dihydroxykaur-16-en-19-oic acid (**33**) (170 mg) in THF (6 ml) was treated overnight with activated MnO_2 (1.7 g) [18]. The reaction mixture was filtered through Celite and evapd under N_2. The Me_2CO-soluble product was *ent*-13-hydroxy-15-oxokaur-16-en-19-oic acid (**32**) (50 mg) which was crystallized from EtOAc–petrol, mp 197–200°; EIMS (probe) 70 ev, *m/z* (rel. int.): 332 $[M]^+$ (18), 304 (14), 294 (25), 277 (100), 260 (25), 167 (38), 121 (56) and 109 (80). Starting material (45 mg) was recovered from the Me_2CO-insoluble fraction.

(*d*) *Reduction of* ent-13-*hydroxy*-15-*oxokaur*-16-*en*-19-*oic acid* (**32**) *with sodium trimethoxyborohydride.* The ketone (**32**) (5 mg) in MeOH (1 ml) at 0° was treated with $NaBH_4$ (6 mg) in MeOH (1 ml) for 1 hr. Water (3 ml) was added and the soln, after adjustment to pH 3.0, was extracted with EtOAc (3 × 3 ml). The product crystallized from EtOH, mp 245–247° and was identified (TLC, GC and MS) as *ent*-13,15β-dihydroxykaur-16-en-19-oic acid (**33**).

The same result was obtained by reduction with $NaBH_4$ in *n*-PrOH at −5°, with $NaBH_4$–Ce^{III}-chloride in EtOH at −70°

and with Al tri-isopropoxide in propan-2-ol.

With $LiAlH_4$ in Et_2O at $-5°$ for 1 hr two products were identified by GC/MS as the MeTMSi ethers: (i) *ent*-13,15β-dihydroxykaur-16-en-19-oic acid (**33**) and (ii) *ent*-13,15β,19-trihydroxykaur-16-ene; *m/z* (rel. int.): 536 $[M]^+$ (15), 521 (5), 446 (14), 431 (7), 407 (12), 281 (96), 244 (58), 229 (100) and 73 (88).

(*e*) ent-13-*Acetoxykaur*-16-*en*-19-*oic acid* (**30**). The 13-hydroxykaurenoic acid (**29**) (500 mg), pyridine (0.2 ml) and Ac_2O (10 ml) were stirred at 80° for 20 hr. The mixture was evapd under vacuum, H_2O (75 ml) and MeOH (25 ml) were added and the product was recovered by extraction with EtOAc (3 × 100 ml). Crystallization from EtOAc gave the required 13-acetate (**30**) (420 mg) mp 178–180° (lit. [10] 199–201°). (Found: C, 73.1; H, 9.2. Calc. for $C_{22}H_{32}O_4$: C, 73.3; H, 9.0%). IR ν_{max}^{nujol} cm^{-1}: 1730, 1695, 1660, 890; ^1H NMR (60 MHz, $CDCl_3$): δ0.98 (3H, *s*, 20-H), 1.23 (3H, *s*, 18-H), 2.00 (3H, *s*, OCOMe), 4.80 (2H, *br s*, 17-H); MS *m/z* (rel. int.): 360 $[M]^+$ (100), 318 (62), 300 (72), 285 (20), 254 (18), 185 (12), 146 (26), 134 (24), 132 (33), 121 (50), 107 (35) and 43 (96).

(*f*) ent-13-*Acetoxy*-15β-*hydroxykaur*-16-*en*-19-*oic acid* (**28**). The acid **30** (300 mg) was treated with SeO_2 (60 mg) and *t*-BuO_2H (210 μl) in $CHCl_3$ (5 ml) for 24 hr. The soln was filtered through Celite which was washed with Me_2CO and the solvents were removed *in vacuo*. *ent*-13-Acetoxy-15β-hydroxykaur-16-en-19-oic acid (**28**) was purified by flash CC [19] using EtOAc–petrol–$MeCO_2H$ (40:60:1) and crystallized from EtOAc as plates, mp 206–208°. (Found: C, 70.3; H, 8.7. $C_{22}H_{32}O_5$ requires C, 70.2; H, 8.6%). IR ν_{max}^{nujol} cm^{-1}, 3500, 1725 and 920; ^1H NMR (90 MHz, $CDCl_3$): δ0.98 (3H, *s*, 20-H), 1.26 (3H, *s*, 18-H), 2.03 (3H, *s*, OCOMe), 3.75 (1H, *s*, 15-H), 5.09 and 5.34 (2H, each *br s*, 17-H); EIMS (probe) 70 eV, *m/z* (rel. int.); 376 $[M]^+$ (abs), 317 (23), 316 (100), 301 (17), 298 (14), 270 (16), 163 (13), 162 (16), 161 (11), 150 (26), 148 (31), 123 (12), 121 (17), 105 (11) and 43 (31).

(*g*) ent-13-*Acetoxy*-15-*oxokaur*-16-*en*-19-*oic acid* (**31**). The unpurified product (440 mg) from (*f*) in Me_2CO (10 ml) was stirred at 0° for 15 min with excess Jones reagent. MeOH (1 ml) and H_2O (50 ml) were added and the organic solvent was removed *in vacuo*. Recovery of the product in EtOAc (3 × 50 ml) gave a gum (338 mg) which crystallized from EtOAc to provide *ent*-13-acetoxy-15-oxokaur-16-en-19-oic acid (**31**), mp 214–216°. (Found: C, 69.9; H, 8.2. $C_{22}H_{30}O_5$ requires C, 70.6; H, 8.1%). IR ν_{max}^{nujol} cm^{-1}: 1730, 1710, 1655 and 940 cm^{-1}; ^1H NMR (90 MHz, $CDCl_3$): δ1.06 (3H, *s*, 20-H), 1.27 (3H, *s*, 18-H), 2.09 (3H, *s*, OAc), 3.18 (1H, *br d*, 9-H), 5.41 and 6.07 (2H, each *s*, 17-H); uv λ_{max}^{MeOH} nm (ε): 230 (6,000); EIMS (probe) 70 eV, *m/z* (rel. int.): 374 $[M]^+$ (abs), 356 (1), 333 (21), 332 (100), 317 (15), 314 (14), 287 (22), 286 (81), 268 (12), 178 (14), 167 (21), 147 (21), 132 (26), 121 (35), 120 (36), 119 (40), 118 (44), 109 (25), 107 (17), 105 (25), and 43 (92).

(*h*) *Reduction of* ent-13-*acetoxy*-15-*oxokaur*-16-*en*-19-*oic acid* (**31**). $NaBH_4$ (45 mg) was stirred in MeOH (4 ml) for 15 min and added to a stirred soln of *ent*-13-acetoxy-15-oxokaur-16-en-19-oic acid (**31**) (115 mg) in MeOH (5 ml). After 48 hr, H_2O (25 ml) was added and the pH adjusted to 3. Extraction with EtOAc (3 × 40 ml), washing with H_2O (20 ml) and removal of the solvent *in vacuo* gave the product which was separated by flash CC [19] using EtOAc–petrol–$MeCO_2H$ (20:80:1), (30:70:1), (50:50:1) then (100:0:1). The first compound eluted was *ent*-13-acetoxy-15α-hydroxykaur-16-en-19-oic acid (**27**) (35 mg), mp 202–204°, which crystallized from EtOAc. (Found: C, 69.3; H, 8.8. $C_{22}H_{32}O_5$ requires C, 70.2; H, 8.6%). IR ν_{max}^{nujol} cm^{-1}: 3200 *br*, 1735, 1720 and 950; ^1H NMR (90 MHz, $CDCl_3$): δ1.02 (3H, *s*, 20-H), 1.27 (3H, *s*, 18-H), 2.03 (3H, *s*, OAc), 4.03 (1H, *s*, 15-H), 5.04 and 5.16 (2H, each *br s*, 17-H); EIMS (probe) 70 eV, *m/z* (rel. int.): 376 $[M]^+$ (6), 317 (23), 316 (100), 288 (14), 270 (8), 260 (3), 173 (6), 167 (6), 164 (7), 150 (13), 122 (16), 121 (23), 120 (14), 105 (9), and 43 (21).

The second compound eluted was *ent*-13-acetoxy-15β-hydroxykaur-16-en-19-oic acid (**28**) (21 mg). Later fractions were shown by GLC to contain a mixture of compounds including the deacetylated analogues **26** and **33** of the compounds isolated from the column.

(i) ent-13,15α-*Dihydroxykaur*-16-*en*-19-*oic acid* (**26**). *ent*-13-Acetoxy-15α-hydroxykaur-16-en-19-oic acid (**27**) (19 mg) in MeOH (10 ml) was treated with K_2CO_3 (500 mg) overnight. The reaction mixture in H_2O (50 ml) was acidified to pH 3 and extracted with EtOAc (3 × 50 ml). After washing with H_2O (20 ml) the solvent was removed *in vacuo* yielding a gum (20 mg). Recrystallization from MeOH–H_2O gave *ent*-13,15α-dihydroxykaur-16-en-19-oic acid (**26**) as needles, mp 190–191°; (Found: M^+ at *m/z* 344.2167. $C_{20}H_{30}O_4$ requires M^+ at *m/z* 334.2144); ^1H NMR (200 MHz, CD_3COCD_3): δ1.00 (3H, *s*, 20-H), 1.21(3H, *s*, 18-H), 3.86 (1H, *br s*, 15-H), and 5.09 (2H, *br s*, 17-H); EIMS (probe) 70 eV, *m/z* (rel. int.): 334 $[M]^+$ (100), 317 (11), 316 (45), 301 (18), 298 (11), 278 (16), 277 (63), 270 (17), 237 (29), 235 (40), 221 (29), 189 (20), 167 (33), 149 (27), 148 (34), 137 (71), 123 (46), 121 (42) and 109 (36); GC/MS (MeTMSi), *m/z* (rel. int.); 492 $[M]^+$ (21), 477 (3), 402 (24), 387 (9), 363 (26), 312 (4), 281 (76), 244 (47), 229 (100), 167 (14), 147 (12) and 73 (75).

GC-MS analysis of the crude product indicated the presence of two other compounds in small amounts (< 10%) as their MeTMSi derivatives; (i) *ent*-13,15β-dihydroxy-16ξ-H-kauran-19-oic acid; *m/z* (rel. int.) 494 $[M]^+$ (6), 404 (17), 364 (81), 363 (100), 351 (6), 305 (20), 235 (14), 195 (19), 191 (35), 182 (28), 121 (24), and 73 (11); and (ii) *ent*-13-hydroxy-15-oxo-16ξ-H-kauran-19-oic acid (**37**). The latter compound was isolated as described in the next section.

Acid treatment of ent-13,15α-*dihydroxykaur*-16-*en*-19-*oic acid* (**20**). The acid (**20**) (6 mg) in MeOH (2 ml) and 2 M HCl (2 ml) was left overnight at room temp. H_2O (10 ml) was added and the product, extracted with EtOAc (3 × 10 ml), was crystallized from Me_2CO–petrol to give *ent*-13-hydroxy-15-oxo-16β-H-kauranoic acid (**37**) (5 mg) mp 132–136°. (Found: M^+ at *m/z* 334.2136; $C_{20}H_{30}O_4$ requires 334.2144); ^1H NMR (200 MHz acetone-d_6): δ1.00 (*d*, *J* = 7 Hz, 17-H_3), 1.02 (*s*, 20-H_3), 1.24 (*s*, 18-H_3); EIMS (probe) 70 eV, *m/z* (rel. int.): 334 $[M]^+$ (100), 291 (10), 288 (11), 277 (88), 167 (33), 123 (39), 121 (38) and 109 (29).

Acid treatment of ent-15α-*hydroxykaur*-16-*en*-19-*oic acid* (**19**). A soln of the acid (100 mg) in EtOH (6 ml) and H_2O (8 ml) containing conc. H_2SO_4 (0.8 ml) was heated for 1 hr under reflux. The soln was cooled in ice, then extracted with EtOAc (3 × 30 ml). The recovered product was purified by flash chromatography with EtOAc–petrol–AcOH (20:80:1 then 30:70:1) to give *ent*-15-oxo-16β-H-kauran-19-oic acid (**36**) (54 mg), mp 205–206.5° (from EtOAc) (lit. [21] 226–268°). (Found: C, 75.6; H, 9.5. Calc. for $C_{20}H_{30}O_3$: C, 75.4; H, 9.5%). IR ν_{max}^{nujol} 1730, 1690 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ1.01 (*s*, 20-H_3), 1.10 (*d*, *J* = 7 Hz, 17-H_3), 1.26 (*s*, 18-H_3) and 2.22 (*q*, *J* = 7 Hz, 16-H); EIMS (probe) 70 eV, *m/z* (rel. int.): 318 $[M]^+$ (48), 275 (15), 260 (100), 245 (25), 123 (16), 121 (22), 109 (18), 107 (21) and 105 (12).

GC/MS data for compounds from H. annuus *seeds and from incubations of* ent-15α-*hydroxykaur*-16-*en*-19-*oic acid* (**19**) with Gibberella fujikuroi, *mutant* B1-41*a* (see Table 1 for occurrence). GA_1 MeTMSi: K_I 2663, *m/z* (rel. int.): 506 $[M]^+$ (100), 491 (9), 448 (12), 377 (12), 375 (10), 235 (7), 207 (21), 193 (8), 167 (6), 131 (9) and 129 (8). GA_4 MeTMSi: K_I 2494, *m/z* (rel. int.): 418 $[M]^+$ (19), 386 (15), 328 (19), 296 (14), 289 (88), 284 (86), 261 (22), 233 (42), 225 (65), 224 (64) and 129 (70).

GA_{19} MeTMSi: K_I 2591, *m/z* (rel. int.): 462 $[M]^+$ (7), 434 (100), 405 (12), 402 (37), 374 (62), 345 (23), 239 (32) and 208 (31).

GA_{20} MeTMSi: K_I 2476, *m/z* (rel. int.): 418 $[M]^+$ (100), 403

(19), 389 (4), 375 (53), 359 (14), 301 (14), 298 (10), 235 (11), 207 (38) and 193 (12).

GA_{45} MeTMSi: K_I 2478, *m/z* (rel. int.): 418 $[M]^+$ (100), 403 (14), 358 (36), 284 (17), 269 (18), 225 (18), 207 (13) and 156 (52).

GA_{63} MeTMSi: K_I 2696, *m/z* (rel. int.): 506 $[M]^+$ (100), 491 (34), 446 (42), 416 (24), 287 (46), 282 (42), 223 (26), 207 (14), 156 (70), and 129 (24).

GA_{64} MeTMSi: K_I 2729, *m/z* (rel. int.): 432 $[M]^+$ (16), 417 (13), 400 (8), 372 (10), 282 (14), 237 (11), 225 (8), 181 (9) and 156 (100).

GA_{65} MeTMSi: K_I 2561, *m/z* (rel. int.): 462 $[M]^+$ (9), 447 (24), 34 (13), 430 (17), 402 (67), 374 (52), 341 (20), 312 (51), 284 (100), 225 (46) and 156 (100).

GA_{66} MeTMSi: K_I 2545, *m/z* (rel. int.): 492 $[M]^+$ (6), 477 (17), 460 (12), 432 (100), 400 (85), 372 (91), 342 (36), 310 (45), 282 (45), 223 (30) and 156 (74).

GA_{67} MeTMSi: K_I 2613, *m/z* (rel. int.): 506 $[M]^+$ (88), 491 (24), 462 (24), 447 (9), 416 (100), 389 (16), 372 (21), 357 (21), 313 (16), 295 (20), 257 (13), 229 (15) and 205 (13).

GA_{72} MeTMSi: K_I 2822, *m/z* (rel. int.): 594 $[M]^+$ (79), 579 (28), 550 (24), 504 (100), 465 (15), 370 (19), 355 (11), 311 (21), 295 (26), 257 (17) and 229 (13).

15β-Hydroxy GA_{12} MeTMSi: K_I 2450, *m/z* (rel. int.): 448 $[M]^+$ (3), 433 (12), 416 (90), 401 (10), 388 (52), 373 (10), 326 (23), 298 (38), 284 (14), 239 (32) and 156 (100).

15β-Hydroxy GA_{13} MeTMSi: K_I 2746, *m/z* (rel. int.): 580 $[M]^+$ (81), 565 (22), 520 (21), 490 (51), 418 (38), 328 (25), 269 (44), 221 (39), 156 (41) and 129 (100).

15β-Hydroxy GA_{14} MeTMSi: K_I 2672, *m/z* (rel. int.): 536 $[M]^+$ (3), 521 (16), 504 (38), 476 (15), 414 (15), 387 (22), 375 (16), 296 (18), 285 (20), 237 (14) and 156 (100).

15β-OH GA_{17} MeTMSi: K_I 2636, *m/z* (rel. int.): 580 $[M]^+$ (22), 565 (10), 548 (10), 521 (19), 460 (22), 430 (42), 371 (35), 295 (100), 281 (17), 244 (30), and 229 (62).

15β-OH GA_{19} MeTMSi: K_I 2671, *m/z* (rel. int.): 550 $[M]^+$ (15), 535 (27), 522 (88), 490 (31), 432 (63), 400 (51), 372 (100), 296 (67), 282 (43), 244 (39), and 229 (92).

15β-OH GA_{44} MeTMSi: K_I 2855, *m/z* (rel. int.): 520 $[M]^+$ (54), 505 (23), 430 (59), 371 (21), 295 (44), 255 (33), 244 (30), 229 (100), and 191 (40).

15β-OH GA_{53} MeTMSi: K_I 2568, *m/z* (rel. int.): 536 $[M]^+$ (37), 521 (16), 504 (12), 492 (21), 476 (43), 446 (31), 432 (24), 414 (37), 386 (49), 296 (83), 244 (100) and 229 (96).

Dioxindole 3-acetic acid MeTMSi: K_I 1872, *m/z* (rel. int.): 293 $[M]^+$ (32), 278 (100), 250 (18), 234 (39), 220 (100), 204 (48), 172 (42) and 89 (69).

Abscisic acid Me ester: K_I 2026, *m/z* (rel. int.): 278 $[M]^+$ (1), 260 (3), 222 (2), 190 (100), 162 (20), 134 (19), 125 (31), 112 (9) and 91 (8).

trans-Abscisic acid Me ester: K_I 2103, *m/z* (rel. int.): 278 $[M]^+$ (0.5), 260 (2), 222 (7), 190 (100), 162 (19), 134 (20). 125 (8) and 91 (9).

Dihydrophaseic acid MeTMSi: K_I 2150, *m/z* (rel. int.): 368 $[M]^+$ (1), 353 (4), 278 (4), 220 (11), 188 (24), 159 (70), 125 (28), 122 (45), 117 (39) and 43 (100).

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