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MICROBIOLOGICAL CONVERSION OF 12-OXYGENATED AND OTHER DERIVATIVES OF *ENT*-KAUR-16-EN-19-OIC ACID BY *GIBBERELLA FUJIKUROI*, MUTANT B1-41a

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Key Word Index—*Gibberella fujikuroi*; fungi; C_{19}- and C_{20}-gibberellins; *ent*-kaurenoids; 11- and 12-oxygenated derivatives; grandiflorenic acid; 12β-hydroxygrandiflorenic acid; attractyligen; *ent*-15β-hydroxykaur-16-en-19-oic acid.

Abstract—The metabolism of several ring C and D-functionalized *ent*-kaur-16-en-19-oic acids by cultures of *Gibberella fujikuroi*, mutant B1-41a, to the corresponding derivatives of the normal fungal gibberellins (GAs) and *ent*-kaurenoids is described. A range of 12α- and 12β-hydroxyGAs and *ent*-kaurenoids are characterized by their mass spectra and GC Kovats retention indices. The mass spectral and GC data are used to identify the 12α-hydroxy derivatives of GA_{12}, GA_{14}, GA_{37} and GA_4 (GA_{58}), and of the 12β-hydroxy derivatives of *ent*-7α-hydroxy- and *ent*-6α,7α-dihydroxykaurenoic acids, in seeds of *Cucurbita maxima*. Similarly the metabolites of GA_9, formed in seeds of *Pisum sativum* and cultures of *G. fujikuroi*, mutant B1-41a, are identified as 12α-hydroxyGA_9. *ent*-11β-Hydroxy- and *ent*-11-oxo-kaurenoic acids are metabolized by the fungus to the corresponding 11-oxygenated derivatives of the normal fungal *ent*-kaurenoids and some C_{20}-GAs; no 11-oxygenated C_{19}-GAs are formed. Grandiflorenic acid, 11β-hydroxygrandiflorenic acid, attractyligen and *ent*-15β-hydroxykaurenoic acid are metabolized to unidentified products.

INTRODUCTION

The preceding paper [1] described the GC/MS of several new gibberellins (GAs) and *ent*-kaurenoids in extracts of seeds of *Cucurbita maxima*. From their mass spectra, the structures were tentatively deduced to be 12-hydroxy analogues of the normal metabolites of *Gibberella fujikuroi*. To establish the structures of these constituents from *C. maxima* and to confirm the 12α-hydroxy structure (**1**), assigned previously to a metabolite of GA_9 (**2**), formed in seedlings [2] and seeds [3] of *Pisum sativum* and in cultures of *G. fujikuroi* mutant B1-41a [4], the microbiological conversion of *ent*-12β-hydroxy-, *ent*-12α-hydroxy- and *ent*-12-oxo-kaurenoic acids (**8**, **9** and **10**, respectively) was investigated using the mutant B1-41a of *G. fujikuroi*. This mutant is blocked [5] for GA-biosynthesis at the step between *ent*-kaurenal (**15**) and *ent*-kaurenoic acid (**16**). Thus, in the near absence of metabolites from the acid (**16**), metabolites from analogues of the acid (**16**) can be readily identified [6–9].

This paper also briefly describes the metabolism of the following additional ring C and D derivatives of *ent*-kaurenoic acid (**16**), the *ent*-11β-alcohol (**11**), the 11-ketone (**12**), *ent*-kaur-9(11),16-dien-19-oic acid (grandiflorenic acid) (**18**), *ent*-12α-hydroxykaur-9(11),16-dien-19-oic acid (**19**), *ent*-2α,15β-dihydroxy-18-norkaur-16-en-19-oic acid (**22**) and *ent*-15β-hydroxykaur-16-en-19-oic acid (**23**).

RESULTS AND DISCUSSION

Resuspension cultures of *G. fujikuroi*, mutant B1-41a, were used. After incubation with a substrate, the extract from the culture filtrate was methylated and trimethylsilylated. The MeTMSi derivatives were analysed by GC/MS using packed and capillary GC columns. The structures of the metabolites were either determined by comparison of the mass spectra with reference spectra of known compounds or they were deduced from the mass spectral fragmentation data of the metabolites and from the structures of the substrates.

12-*Oxygenated substrates*

The compounds **8**, **9** and **10** were prepared as described previously [10]. A crucial step in the sequence from grandiflorenic acid (**18**) was the catalytic reduction of the diketone (**20**). Support for the 9βH-stereochemistry in the reduction product **13** was obtained by oxidation of *ent*-12β-hydroxykaurenoic acid (**8**) to the norketone (**14**) which, like the norketone (**17**) from *ent*-kaurenoic acid (**16**), showed a positive CD curve. Subsequently this stereochemistry was conclusively established by the more convenient isolation of *ent*-12β-hydroxykaurenoic acid (**8**) from *Helianthus decapetalus* [11, 12] and its 12-deoxygenation to *ent*-kaurenoic acid (**16**).

Incubation of *ent*-12β-hydroxykaurenoic acid (**8**) with resuspended mycelium of *G. fujikuroi*, mutant B1-41a, was conducted both at pH 4.8 and at pH 6.6. The MeTMSi derivatives of the unseparated neutral and acidic metabolites were analysed by GC/MS on packed columns (2% QF-1 and 2% SE-33) and the separated acidic metabolites on a WCOT capillary column (OV-1). The following metabolites were identified from incubations at pH 4.8: 12α-hydroxyGA_{12} (**25**), 12α-hydroxyGA_{14} (**26**), GA_{39} (**36**), 12α-hydroxy GA_4 (GA_{58}) (**3**), *ent*-7α,12β-dihydroxykaurenoic acid (**46**), *ent*-6α,7α,12β-trihydroxykaurenoic

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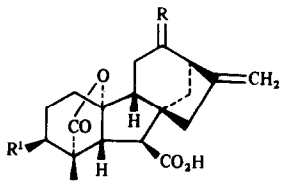
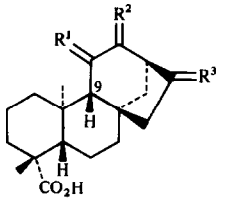
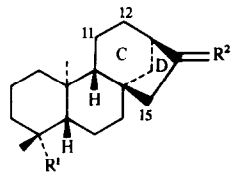


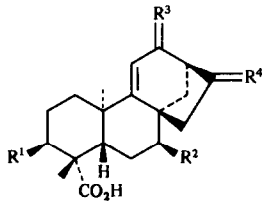
1 R^1=H, R^2=H,αOH
2 R^1=H, R^2=H_2
3 R^1=OH, R^2=H,αOH
4 R^1=H, R^2=H,βOH
5 R^1=OH, R^2=H,βOH
6 R^1=H, R^2=O
7 R^1=OH, R^2=O



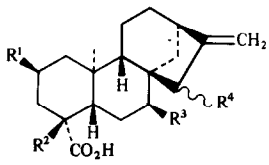
	R^1	R^2	R^3
8	H_2	H,αOH	CH_2
9	H_2	H,βOH	CH_2
10	H_2	O	CH_2
11	H,αOH	H_2	CH_2
12	O	H_2	CH_2
13	H_2	O	O
14	H_2	H,αOH	O



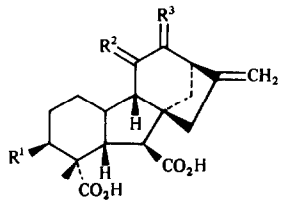
15 R^1=CHO, R^2=CH_2
16 R^1=CO_2H, R^2=CH_2
17 R^1=CO_2H, R^2=O



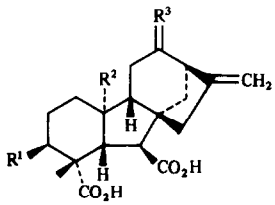
	R^1	R^2	R^3	R^4
18	H	H	H_2	CH_2
19	H	H	H,βOH	CH_2
20	H	H	O	O
21	OH	OH	H_2	CH_2



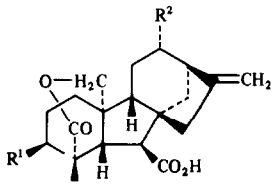
	R^1	R^2	R^3	R^4
22	OH	H	H	αOH
23	H	Me	H	αOH
24	H	Me	H	βOH



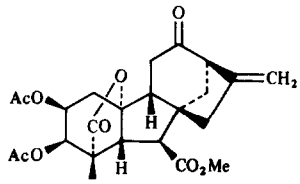
	R^1	R^2	R^3
25	H	H_2	H,αOH
26	OH	H_2	H,αOH
27	H	H_2	H,βOH
28	OH	H_2	H,βOH
29	H	H_2	O
30	OH	H_2	O
31	H	H,αOH	H_2
32	OH	H,αOH	H_2
33	H	O	H_2
34	OH	O	H_2



	R^1	R^2	R^3
35	OH	CHO	H,αOH
36	OH	CO_2H	H,αOH
37	OH	CO_2H	H,βOH
38	H	CHO	O
39	OH	CO_2H	O
40	H	CHO	H,βOH
41	OH	CHO	H,βOH
42	H	CO_2H	H,αOH



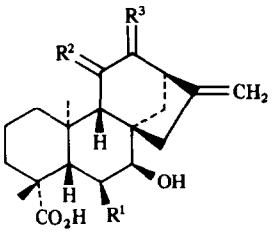
43 R^1=OH, R^2=H,αOH
44 R^1=H, R^2=H,βOH



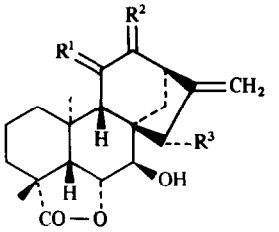
45

acid (**47**) and 7β,12α-dihydroxykaurenolide (**54**). In addition weak mass spectra were observed which can be tentatively assigned to the MeTMSi derivatives of 12α-hydroxyGA_{36} (**35**) and 12α-hydroxyGA_{37} (**43**). The metabolites from incubations at pH 6.6 were qualitatively identical except that 7β,12α-dihydroxykaurenolide (**54**) was absent. Thus mainly 3-hydroxylated metabolites were formed from *ent*-12β-hydroxykaurenoic acid (**8**) at both pH values. The absence of 12α-hydroxyGA_9 (**1**), especially in the pH 6.6 incubations was surprising since Bearder *et al.* [13, 14] found that GA_9 (**2**) and 12,16-cyclo GA_9 were formed at the higher pH by incubation of GA_{12} and trachylobanic acid respectively.

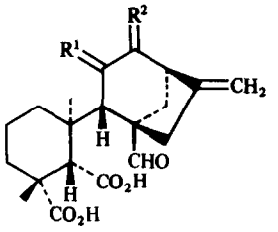
The metabolism of *ent*-12α-hydroxykaurenoic acid (**9**)



	R^1	R^2	R^3
46	H	H_2	H,αOH
47	OH	H_2	H,αOH
48	OH	H_2	H,βOH
49	H	H_2	H,βOH
50	OH	H_2	O
51	OH	H,αOH	H_2
52	H	H,αOH	H_2
53	OH	O	H_2



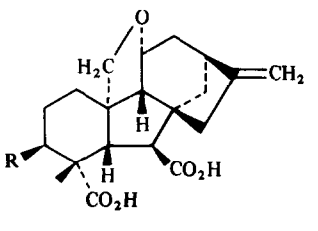
	R^1	R^2	R^3
54	H_2	H,αOH	H
55	H_2	H,βOH	H
56	H_2	O	H
57	O	H_2	H
58	H_2	H_2	OH



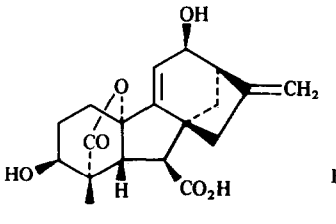
59 $R^1 = H_2$, $R^2 = O$

60 $R^1 = H,\alpha OH$; $R^2 = H_2$

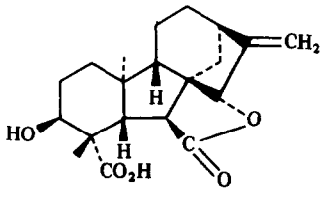
61 $R^1 = O$, $R^2 = H_2$



	R
62	H
63	OH



64



65

was examined as for the *ent*-12β-epimer (**8**). The products from the incubations at pH 4.8 were the 12β-hydroxy derivatives **5**, **4**, **27**, **28**, **44**, **40**, **41**, **48** and **55** of GA_4, GA_9, GA_{12}, GA_{14}, GA_{15}, GA_{24}, GA_{36}, *ent*-6α,7α-dihydroxykaurenoic acid and 7β-hydroxykaurenolide, respectively. At pH 6.6 the metabolites were the same as at pH 4.8 except for the absence of the kaurenolide (**55**) and the presence of *ent*-7α,12α-dihydroxykaurenoic acid (**49**).

The 12α- and 12β-epimers of each metabolite cannot be distinguished from each other by their mass spectra. However, they do have different GC-retention times (Table 1). Thus the constituents in extracts of seeds of *C. maxima* described in the previous paper [1], were shown to be the 12α-hydroxy derivatives **3**, **25**, **26** and **43** of GA_4, GA_{12}, GA_{14} and GA_{37}, respectively, and the 12β-hydroxy derivatives **49** and **48** of *ent*-7α-hydroxy- and -6α-7α-dihydroxykaurenoic acids, respectively. The GC retention times of the 12α- and 12β-hydroxykaurenolides **54** and **55** are very close (Table 1) but co-injections identified the kaurenolide in *C. maxima* as the 12α-hydroxy epimer (**54**). The isolation, and full characterization, of 12α-hydroxyGA_4 (GA_{58}) (**3**) and of *ent*-6α,7α,12α-trihydroxykaurenoic acid (**48**), from seeds of *C. maxima* is described in the following paper [15]. Wada *et al.* [16] have recently reported, without experimental details, that cultures of *G. fujikuroi* blocked for GA-biosynthesis with chlorocholine chloride, transformed *ent*-12β-hydroxykaurene into five main metabolites tentatively identified as **25**, **42**, **43**, **46** and **47**.

Attention was next directed to the identification of the metabolite of GA_9 (**2**), formed in seeds of *Pisum sativum* [3]. The MeTMSi derivatives of the metabolite and of 12β-hydroxyGA_9 (**4**) were found to have very similar mass spectra, but different GC-retention times, supporting the original suggestion [3] that the metabolite was the epimeric 12α-hydroxyGA_9 (**1**). Since 12α-hydroxyGA_9 (**1**) was not formed from the incubation of *ent*-12β-hydroxykaurenoic acid (**8**) by cultures of *G. fujikuroi*, mutant B1-41a (see earlier), an alternative preparation of this compound was sought. The preparation of 12-oxoGA_9 (**6**) by incubation of *ent*-12-oxokaurenoic acid (**10**) with the fungal mutant was therefore investigated with the view to the subsequent chemical reduction of 12-oxoGA_9 (**6**) to 12α-hydroxyGA_9 (**1**). An analogy for the reduction step is provided by the work of Yokota *et al.* [17] who have shown that reduction of GA_{26} diacetate methyl ester (**45**) yielded both the 12α- and 12β-alcohols.

ent-12-Oxokaurenoic acid (**10**) was incubated with *G. fujikuroi*, mutant B1-41a. The formation of the following metabolites was deduced by GC/MS using a WCOT OV-1 capillary column: 12-oxoGA_{12} (**29**), 12-oxoGA_{13} (**39**), 12-oxoGA_{14} (**30**), 12-oxoGA_{24} (**38**), 12-oxoGA_9 (**6**), 12-oxoGA_4 (**7**), *ent*-6α,7α-dihydroxy-12-oxokaurenoic acid (**50**), 7β-hydroxy-12-oxokaurenolide (**56**) and the di-acid

Table 1. Retention time data for MeTMSi derivatives of 12-hydroxy metabolites

Compound	R_t (min. sec.)	Kovats RI	ΔRR_t
12α-hydroxyGA$_4$ (GA$_{58}$) (3)	31:38	2727	$C_{27}+0{:}36$
12β-hydroxy GA$_4$ (5)	30:39	2683	$C_{26}+1{:}51$
12α-hydroxyGA$_9$ (1)	27:26	2541	$C_{25}+0{:}56$
12β-hydroxyGA$_9$ (4)	26:14	2489	$C_{24}+2{:}07$
12α-hydroxyGA$_{12}$ (25)	27:22	2538	$C_{25}+0{:}52$
12β-hydroxyGA$_{12}$ (27)	27:42	2553	$C_{25}+1{:}12$
12α-hydroxyGA$_{13}$ (GA$_{39}$) (36)	32:31	2769	$C_{27}+1{:}29$
12β-hydroxyGA$_{13}$ (37)	33:01	2791	$C_{27}+1{:}50$
12α-hydroxyGA$_{14}$ (26)	30:39	2683	$C_{26}+1{:}51$
12β-hydroxyGA$_{14}$ (28)	31:11	2707	$C_{27}+0{:}09$
12α-hydroxyGA$_{36}$ (35)	32:42	2777	$C_{27}+1{:}40$
12β-hydroxyGA$_{36}$ (36)	32:54	2786	$C_{27}+1{:}52$
12α-hydroxyGA$_{37}$ (43)	36:20	2953	$C_{29}+1{:}04$
ent-7α,12β-dihydroxykaurenoic acid (49)	27:55	2562	$C_{25}+1{:}25$
ent-7α,12α-dihydroxykaurenoic acid (46)	29:08	2615	$C_{26}+0{:}20$
ent-6α,7α,12β-trihydroxykaurenoic acid (47)	31:20	2714	$C_{27}+0{:}18$
ent-6α,7α,12α-trihydroxykaurenoic acid (48)	32:31	2769	$C_{27}+1{:}29$
7α,12α-dihydroxykaurenolide (54)	30:23	2671	$C_{26}+1{:}35$
7β,12β-dihydroxykaurenolide (55)	30:38	2682	$C_{26}+1{:}50$

(59). Reduction of part of the total acidic metabolites from the 12-ketone (10) with sodium borohydride gave a complex mixture. Capillary GC/MS of the MeTMSi derivatives indicated the possible presence of 12α-hydroxyGA$_9$ (1) with the same retention time as one of the major products, identified as 12α-hydroxyGA$_{12}$ (25). The remaining acidic metabolites were separated as the methyl esters by HPLC on a Spherisorb-ODS column which was eluted isocratically with methanol–water (3:2). Three major fractions were collected, reduced with sodium borohydride in THF, and the reduction products were analysed as the TMSi derivatives by capillary GC/MS. In the least polar fraction, 12α- and 12β-hydroxyGA$_{12}$ (25 and 27) were identified. In the fraction of intermediate polarity, *ent*-7α,12β- and *ent*-7α,12α-dihydroxykaurenoic acids (46 and 49) were identified together with several unidentified products with an ion at m/z 506 in their mass spectra. In the most polar fraction 12α- and 12β-hydroxyGA$_9$ (1 and 4), were identified together with an unidentified pair of epimers with an ion at m/z 506. From the reduction of other minor fractions from HPLC the MeTMSi derivatives of 12α- and 12β-hydroxyGA$_{14}$ (26 and 28) and of 12α- and 12β-hydroxyGA$_4$ (3 and 5), were identified together with 12β-hydroxyGA$_{13}$ (12-epiGA$_{39}$, 37).

The Kovats retention indices of the MeTMSi derivatives of 12α- and 12β-hydroxyGA$_9$ (1 and 4) were 2541 and 2489, respectively (Table 1). The Kovats retention index of the MeTMSi derivative of the metabolite of GA$_9$, from seeds of *P. sativum* [3] was 2541. The mass spectra of the MeTMSi derivatives of 12α-hydroxyGA$_9$ (1) and of the GA$_9$-metabolite were identical. On this evidence the structure of the GA$_9$ metabolite in seeds of *P. sativum* was confirmed as 12α-hydroxyGA$_9$ (1).

The 12α-hydroxyGA$_9$ structure (1) has also been tentatively assigned [4] to a minor metabolite from incubation of GA$_9$ (2) with cultures of *G. fujikuroi*, mutant B1-41a. This incubation was repeated and capillary GC/MS of the MeTMSi derivatives of one of the minor acidic metabolites had the same mass spectrum and Kovats retention number (2541) as the MeTMSi derivative of 12α-hydroxyGA$_9$ (1), thereby confirming the structure of this fungal metabolite of GA$_9$.

11-*Oxygenated substrates* [10]

Unlike the 12-oxygenated compounds, the 11-alcohol (11) and the 11-ketone (12) were not metabolized to C$_{19}$-GAs at either pH 4.8 or 6.6. At both pH values, 11α-hydroxyGA$_{12}$ (33) was the major metabolite from the 11-alcohol (11). Smaller amounts of 11α-hydroxyGA$_{14}$ (32), *ent*-6α,7α,11β-trihydroxykaurenoic acid (51) and the diacid (60) were also detected. At pH 6.6, but not at pH 4.8, *ent*-7α,11β-dihydroxykaurenoic acid (52) was formed in minor amount.

The major metabolite from the 11-ketone (12) was 11-oxoGA$_{14}$ (34); minor amounts of 11-oxoGA$_{12}$ (33), 7β-hydroxy-11-oxokaurenolide (57), the diacid (61) and *ent*-6α,7α-dihydroxy-11-oxokaurenoic acid (53) were detected.

The non-formation of C$_{19}$-GAs from the 11-oxygenated substrates may be because of the proximity of the C-11 oxygen function and C-20, and two metabolites of the 11-alcohol (11) were detected in trace amounts which had mass spectra consistent with the 20,11-ethers 62 and 63.

Miscellaneous substrates

Grandiflorenic acid (18) was metabolized to two main products the mass spectrum of which indicated the 3β,7β-dihydroxy derivative 21 and a monohydroxy derivative of 21. No GAs were detected. 12β-Hydroxygrandiflorenic acid (19) was metabolized to many unidentified compounds. The mass spectrum of one of them was consistent with structure 64.

ent-15β-Hydroxykaurenoic acid (23) was also metabolized to many compounds, three of which were tentatively identified from mass spectral data as 7β,15α-dihydroxykaurenolide (58), the 7,15α-lactone (65) and a hydroxy derivative of 65. Thus the metabolism of the *ent*-

15β-epimer (**23**) is different from that of the *ent*-15α-epimer (**24**) which was previously shown [8] to be metabolized to 15β-hydroxy analogous of the normal fungal GAs. Attractyligen (**22**) was completely metabolized to a large number of compounds, but none of the metabolites were identified.

EXPERIMENTAL

Incubation of substrates. The compounds (1–2 mg), in Me_2CO (100 μl) were added to pigmented mycelium of *Gibberella fujikuroi*, mutant B1-41a, obtained from cultures in 40%-N ICI medium (50 ml) in conical flasks (250 ml) and resuspended in 0%-N ICI medium (50 ml) in conical flasks. The resuspension medium was either at the natural pH or buffered at pH 6.6 by the addition of KH_2PO_4 (1.36 g) and the appropriate vol. of aq. KOH soln. The cultures were shaken for 5 days at 25° on a reciprocal shaker. Full details of the procedure are given in ref. [4].

Analysis of metabolites. After incubation, the culture filtrate was acidified to pH 2.5 with 2 M HCl and extracted with EtOAc. The recovered residue was methylated (CH_2N_2) then trimethylsilylated [18]. The MeTMSi derivatives were analysed by GC/MS using a GEC-AEI MS 30 mass spectrometer and either a 2% QF-1 or 2% SE-33 packed column under the conditions previously described [1]. MS data for the metabolites are listed below. In some experiments, detailed in Results and Discussion, the culture filtrates were adjusted to pH 8.0 with $NaHCO_3$ and extracted with EtOAc. The aq. fraction was then acidified with 2 M HCl to pH 2.5 and extracted with EtOAc to give the acidic metabolites. These acids were methylated (CH_2N_2) and the recovered methyl esters were dissolved in dry CH_2Cl_2 to remove insoluble carbohydrates. The methyl esters recovered from the CH_2Cl_2 soln were trimethylsilylated [8] and the MeTMSi derivatives were analysed by GC/MS using a VG 7050 mass spectrometer and an OV-1 WCOT fused silica column (25 m × 0.2 mm i.d.) under the conditions described in the previous paper.

HPLC of the methyl esters of the metabolites from the 12-*ketone* (**10**) *and reduction of the fractions.* The acidic metabolites from two incubations of *ent*-12-oxo-kaurenoic acid (2 × 2 mg) as detailed earlier were methylated (CH_2N_2). The product was subjected to HPLC on a column (46 × 250 mm) of Spherisorb ODS (10 μ), eluted isocratically with MeOH–H_2O (3:2) at a flow-rate of 1.0 ml/min. Elution was monitored by UV absorption at 209 nm. Three main fractions with $R_1 = 3.6$, $R_2 = 4.9$ and $R_3 = 9.4$, were collected and analysed by capillary GC/MS as described earlier.

Fraction R_1, containing 12-oxoGA_9 the methyl ester of 12-oxoGA_9 (**6**), in THF (4 ml) was stirred for 1 hr with $NaBH_4$ (3 mg). After the addition of H_2O (5 ml), the soln was acidified to pH 3.0 and extracted with EtOAc (3 × 10 ml). The EtOAc extract was washed with H_2O (5 ml) then toluene was added and the solvent was removed *in vacuo*. The residue was trimethylsilylated and subjected to capillary GC/MS under the conditions defined earlier. The two major compounds were the MeTMSi ethers of 12α-hydroxyGA_9 (**1**) (C-number, 2541) and 12β-hydroxyGA_9 (C-number 2489) (for *m/z* values, see later), together with the MeTMSi derivatives of 12-epimeric alcohols with $[M]^+$ at *m/z* 506.

Fraction R_2 was similarly reduced with $NaBH_4$ in THF and analysed by capillary GC/MS. The MeTMSi derivatives of *ent*-7α,12α- and *ent*-7α,12β-dihydroxykaurenoic acids (**49** and **46**) were identified together with several MeTMSi derivatives with $[M]^+$ at *m/z* 506.

Reduction of fraction R_3 and capillary GC/MS of the trimethylsilylated product showed the presence of the MeTMSi derivatives of 12α- and 12β-hydroxyGA_{12} (**25** and **27**). The other fractions from the HPLC column were combined, trimethylsilylated and examined by capillary GC/MS. The MeTMSi derivatives of 12α- and 12β-hydroxyGA_{14} (**26** and **28**), of 12α- and 12β-hydroxyGA_4 (**3** and **5**), and 12β-hydroxyGA_{13} (**37**) were detected.

MS of MeTMSi derivatives of metabolites (*normalized on most intense ion above* m/z 75). (A). From *ent*-12β-hydroxykaurenoic acid (**8**): (a) 12α-HydroxyGA_{12} (**25**), *m/z* (rel. int.): 448 $[M]^+$ (5), 416 (92), 388 (51), 326 (33), 298 (99), 239 (49), 209 (94), 208 (38), 207 (100), 181 (46), 129 (30), 121 (22), 119 (22), 107 (38), 103 (29). (b) 12α-HydroxyGA_{14} (**26**), *m/z* (rel. int.): 536 $[M]^+$ (4), 521 (18), 504 (95), 476 (25), 446 (12), 414 (31), 386 (39), 297 (47), 285 (65), 257 (58), 229 (47), 207 (67), 129 (100). (c) 12α-HydroxyGA_4 (GA_{58}) (**3**), *m/z* (rel. int.): 506 $[M]^+$ (27), 491 (8), 416 (51), 384 (44), 356 (45), 326 (22), 317 (20), 294 (23), 287 (24), 282 (43), 267 (32), 227 (40), 223 (61), 221 (47), 129 (100), 103 (31). (d) 12α-HydroxyGA_{36} (**35**), *m/z* (rel. int.): 550 $[M]^+$ (0), 535 (1), 460 (12), 400 (27), 372 (33), 340 (23), 310 (37), 282 (71), 280 (100), 238 (52), 223 (37), 221 (79), 220 (40), 219 (30), 209 (22), 193 (25), 129 (22). (e) 12α-HydroxyGA_{37} (**43**), *m/z* (rel. int.): 520 $[M]^+$ (10), 505 (3), 430 (14), 340 (14), 319 (16), 271 (16), 217 (100), 204 (99), 191 (17), 182 (10), 147 (18), 129 (100), 103 (17). (f) GA_{39} (**36**), *m/z* (rel. int.): 580 $[M]^+$ (1), 565 (13), 548 (8), 490 (16), 400 (33), 372 (33), 282 (41), 129 (100), 109 (85). (g) *ent*-7α,12β-Dihydroxykaurenoic acid (**46**), *m/z* (rel. int.): 492 $[M]^+$ (1), 477 (1), 402 (18), 312 (39), 297 (19), 286 (18), 253 (24), 252 (27), 237 (13), 221 (20), 191 (24), 183 (15), 144 (28), 131 (21), 129 (100). (h) *ent*-6α,7α,12β-Trihydroxykaurenoic acid (**47**), *m/z* (rel. int.): 580 $[M]^+$ (1), 565 (17), 490 (21), 269 (100), 204 (19), 191 (14), 147 (12), 108 (13). (i) 7β,12α-Dihydroxykaurenolide (**54**), *m/z* (rel. int.): 476 $[M]^+$ (3), 461 (2), 433 (4), 386 (13), 296 (100), 172 (16), 147 (15), 137 (27), 129 (13), 109 (81), 103 (22).

(B). From *ent*-12α-hydroxykaurenoic acid (**9**): (a) 12β-HydroxyGA_{12} (**27**), *m/z* (rel. int.): 448 $[M]^+$ (2), 416 (72), 388 (41), 326 (33), 298 (100), 239 (53), 213 (37), 209 (89), 207 (99), 181 (62), 180 (67), 129 (43), 121 (30), 119 (30), 107 (31), 105 (35), 103 (37). (b) 12β-HydroxyGA_{14} (**28**), *m/z* (rel. int.): 536 $[M]^+$ (2), 521 (6), 504 (30), 476 (14), 209 (31), 143 (30), 137 (33), 129 (100), 117 (31), 109 (33), 105 (31), 103 (40), 89 (31). (c) 12β-HydroxyGA_4 (**5**), *m/z* (rel. int.): 506 $[M]^+$ (26), 491 (19), 474 (10), 461 (62), 384 (75), 372 (45), 357 (47), 356 (86), 326 (35), 317 (37), 313 (43), 294 (45), 287 (34), 282 (79), 281 (37), 280 (39), 267 (62), 266 (73), 253 (36), 240 (30), 223 (100), 221 (85), 197 (67), 195 (40), 181 (35), 129 (59). (d) 12β-HydroxyGA_9 (**4**), *m/z* (rel. int.): 418 $[M]^+$ (26), 403 (22), 386 (11), 372 (22), 296 (61), 283 (44), 282 (44), 268 (100), 257 (48), 225 (57), 223 (94), 217 (41), 197 (52), 182 (44), 180 (74), 149 (74), 143 (41), 129 (56), 103 (50), 91 (41). (e) 12β-HydroxyGA_{15} (**44**), *m/z* (rel. int.): 432 $[M]^+$ (36), 400 (17), 342 (30), 310 (42), 297 (21), 296 (37), 283 (52), 282 (83), 237 (100), 236 (20), 225 (25), 223 (20), 207 (28), 182 (26), 181 (22), 131 (23), 129 (48), 118 (21). (f) 12β-HydroxyGA_{24} (**40**), *m/z* (rel. int.): 462 $[M]^+$ (6), 447 (1), 430 (13), 402 (15), 312 (83), 286 (37), 284 (100), 258 (35), 237 (58), 195 (74), 167 (52), 107 (53). (g) 12β-HydroxyGA_{36} (**41**), *m/z* (rel. int.): 550 $[M]^+$ (13), 535 (10), 490 (26), 460 (23), 400 (78), 372 (78), 342 (28), 310 (65), 299 (52), 282 (100), 256 (45), 223 (57), 197 (33), 185 (30), 129 (57). (h) *ent*-7α,12α-Dihydroxykaurenoic acid (**49**), *m/z* (rel. int.): 492 $[M]^+$ (2), 477 (1), 402 (21), 312 (45), 297 (20), 252 (26), 221 (20), 191 (27), 144 (26), 129 (100). (i) *ent*-6α,7α,12α-Dihydroxykaurenoic acid (**48**), *m/z* (rel. int.): 580 $[M]^+$ (< 1), 565 (11), 269 (100), 209 (22), 191 (21), 147 (14), 129 (12). (j) 7β,12β-Dihydroxykaurenolide (**55**), *m/z* (rel. int.): 476 $[M]^+$ (< 1), 416 (4), 386 (8), 296 (69), 137 (21), 129 (29), 109 (100), 105 (31).

(C). From 12-oxokaurenoic acid (**10**): (a) 12-oxoGA_{12} (**29**), *m/z* (rel. int.): 374 $[M]^+$ (5), 342 (16), 314 (100), 299 (23), 256 (26), 195 (31), 109 (21), 107 (35). (b) 12-OxoGA_{13} (**39**), *m/z* (rel. int.):

506 $[M]^+$ (5), 491 (20), 446 (29), 414 (33), 386 (23), 363 (39), 324 (54), 298 (31), 297 (36), 296 (51), 269 (33), 253 (37), 129 (100). (c) 12-OxoGA$_{14}$ (**30**), *m/z* (rel. int.): 462 $[M]^+$ (2), 447 (5), 430 (5), 416 (8), 402 (8), 312 (14), 273 (11), 129 (100). (d) 12-OxoGA$_{24}$ (**38**), *m/z* (rel. int.): 388 $[M]^+$ (1), 360 (15), 356 (12), 328 (46), 300 (100), 239 (30), 197 (43), 137 (32), 121 (31), 119 (41), 105 (41). (e) 12-OxoGA$_4$ (**7**), *m/z* (rel. int.): 432 $[M]^+$ (55), 417 (19), 404 (41), 259 (20), 242 (25), 312 (20), 303 (16), 298 (23), 255 (25), 254 (23), 239 (44), 209 (23), 197 (21), 195 (38), 155 (21), 129 (100), 105 (23). (f) 12-OxoGA$_9$ (**6**), *m/z* (rel. int.): 344 $[M]^+$ (17), 316 (37), 313 (11), 312 (7), 257 (100), 256 (40), 197 (69), 107 (55), 106 (42), 105 (25). (g) *ent*-6α,7α-Dihydroxy-12-oxokaurenoic acid (**50**), *m/z* (rel. int.): 506 $[M]^+$ (21), 491 (47), 416 (31), 273 (22), 269 (100), 209 (27), 147 (39). (h) *ent*-6,7-seco-7-Oxokaur-16-ene-7,19-dioic acid (**59**), (di-Me ester), *m/z* (rel. int.): 390 $[M]^+$ (1), 372 (4), 213 (19), 195 (41), 167 (50), 135 (14), 109 (27), 107 (100). (i) 7β-Hydroxy-12-oxokaurenolide (**56**), *m/z* (rel. int.): 402 $[M]^+$ (< 1), 359 (8), 312 (22), 137 (100), 109 (56).

(D). From *ent*-11β-hydroxykaurenoic acid (**11**): (a) 11α-HydroxyGA$_{12}$ (**31**), *m/z* (rel. int.): 448 $[M]^+$ (13), 388 (10), 345 (28), 298 (13), 239 (16), 208 (11), 207 (15), 167 (100), 117 (14), 107 (16). (b) 11α-HydroxyGA$_{14}$ (**32**), *m/z* (rel. int.): 536 (19), 521 (11), 446 (11), 347 (19), 343 (18), 347 (19), 343 (18), 285 (27), 257 (27), 109 (11), 168 (11), 167 (26), 149 (54), 129 (100). (c) *ent*-7α,11β-Dihydroxykaurenoic acid (**52**), *m/z* (rel. int.): 492 $[M]^+$ (19), 402 (63), 312 (17), 311 (25), 253 (22), 221 (49), 209 (48), 195 (27), 193 (100), 131 (27), 107 (20). (d) *ent*-6α,7α,11β-Trihydroxykaurenoic acid (**51**), *m/z* (rel. int.): 580 $[M]^+$ (2), 565 (8), 269 (57), 209 (21), 168 (10), 167 (12), 109 (100), 107 (23), 79 (11). (e) Unknown, possibly 11α-hydroxyGA$_{12}$ 11, 20-lactone (**62**), *m/z* (rel. int.): 376 (1), 342 (18), 314 (100), 284 (28), 255 (16), 225 (65), 183 (10), 131 (13).

(E). From 11-oxokaurenoic acid (**12**): (a) 11-OxoGA$_{12}$ (**33**), *m/z* (rel. int.): 374 $[M]^+$ (1), 314 (100), 299 (13), 255 (25), 239 (10), 159 (11). (b) 11-OxoGA$_{14}$ (**34**), *m/z* (rel. int.): 462 $[M]^+$ (< 1), 447 (4), 405 (12), 402 (4), 333 (28), 301 (14), 273 (32), 160 (10), 131 (11), 130 (46), 129 (100). (c) *ent*-6α,7α-Dihydroxy-11-oxokaurenoic acid (**53**), *m/z* (rel. int.): 506 $[M]^+$ (< 1), 491 (24), 416 (15), 269 (59), 209 (27), 181 (52), 182 (100), 147 (26), 103 (17). (d) *ent*-6,7-seco-11-Oxokaurene-6,7,19-trioic acid (**61**), *m/z* (rel. int.): 390 $[M]^+$ (< 1), 347 (3), 302 (3), 195 (55), 167 (73), 135 (28), 109 (21), 107 (100). (e) 7β-Hydroxy-11-oxokaurenolide (**57**), *m/z* (rel. int.): 402 $[M]^+$ (3), 359 (14), 312 (31), 256 (19), 147 (100), 137 (46), 109 (71), 93 (34), 92 (24).

(F). From *ent*-kaur-9(11),16-dien-19-oic acid (**18**): (a) Unknown, possibly *ent*-3α,7α-dihydroxykaur-9(11)-en-19-oic acid (**21**), *m/z* (rel. int.): 490 (2), 475 (19), 400 (29), 385 (31), 325 (22), 310 (56), 295 (43), 273 (34), 271 (28), 251 (29), 235 (52), 211 (100), 207 (20), 199 (22), 197 (59), 193 (33), 183 (28), 173 (22), 169 (29), 157 (22), 143 (25). (b) Unknown, possibly *ent*-3α,7α,18-trihydroxykaur-9(11),16-dien-19-oic acid, *m/z* (rel. int.): 578 (< 1), 563 (2), 488 (10), 475 (2), 398 (26), 383 (34), 275 (51), 273 (100), 143 (30), 133 (31), 129 (41), 103 (37).

(G). From *ent*-12α-hydroxykaur-9(11),16-dien-19-oic acid (**19**): Unknown, possibly 12β-hydroxyGA$_4$-9(11)-ene (**64**), *m/z* (rel. int.): 504 (5), 489 (6), 414 (13), 287 (14), 261 (15), 220 (100), 205 (20), 180 (10), 169 (15), 158 (18), 129 (66), 117 (19), 109 (49), 103 (14), 91 (11).

(H). From *ent*-15β-hydroxykaurenoic acid (**23**): (a) Unknown, possibly GA$_{14}$ 7,15-lactone (**65**), *m/z*: 432, 417, 414, 400, 373, 372, 255 and 129. (b) Unknown, possibly a hydroxy GA$_{14}$ 7,15-lactone, *m/z* (rel. int.): 520 (4), 505 (15), 502 (33), 488 (35), 398 (40), 310 (75), 295 (42), 283 (40), 282 (42), 243 (47), 156 (36), 147 (75), 143 (33), 133 (35), 129 (93), 103 (100). (c) Unknown, possibly 7β,15α-dihydroxykaurenolide (**58**), *m/z* (rel. int.): 476 (8), 386 (45), 156 (100), 147 (17), 109 (50).

(I). From reduction of the acidic metabolites from *ent*-12-oxokaurenoic acid (**10**). In addition to the 12α- and 12β-hydroxy metabolites, identified as metabolites of *ent*-12α- and 12β-hydroxykaurenoic acids, the following were identified: (a) 12α-HydroxyGA$_9$ (**1**), *m/z* (rel. int.): 418 $[M]^+$ (10), 386 (11), 372 (17), 284 (26), 283 (45), 284 (49), 268 (100), 251 (29), 225 (39), 223 (92), 209 (17), 197 (21), 182 (16), 169 (12). (b) 12β-HydroxyGA$_{13}$ (12-epiGA$_{39}$) (**37**), *m/z* (rel. int.): 580 $[M]^+$ (0), 565 (11), 520 (15), 488 (30), 43 (39), 398 (65), 371 (33), 370 (42), 308 (43), 282 (21), 281 (31), 280 (41), 221 (32), 129 (100), 119 (21), 107 (22).

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REFERENCES

1. Blechschmidt, S., Castel, U., Gaskin, P., Graebe, J. E., Hedden, P. and MacMillan, J. (1984) *Phytochemistry* **23**, 553.
2. Railton, I. D., Durley, R. C. and Pharis, R. P. (1974) *Plant Physiol.* **54**, 6.
3. Frydman, V. M. and MacMillan, J. (1975) *Planta* **125**, 181.
4. Bearder, J. R., Frydman, V. M., Gaskin, P., Hatton, I. K., Harvey, W. E., MacMillan, J. and Phinney, B. O. (1976) *J. Chem. Soc. Perkin Trans.* 1, 178.
5. Bearder, J. R., MacMillan, J., Wels, C. M., Chaffey, M. B. and Phinney, B. O. (1974) *Phytochemistry* **13**, 911.
6. Bearder, J. R., MacMillan, J., Wels, C. M. and Phinney, B. O. (1975) *Phytochemistry* **14**, 1741.
7. Bearder, J. R., Frydman, V. M., Gaskin, P., MacMillan, J., Wels, C. M. and Phinney, B. O. (1976) *J. Chem. Soc. Perkin Trans.* 1, 173.
8. Bearder, J. R., Dennis, F. G., MacMillan, J., Martin, G. C. and Phinney, B. O. (1975) *Tetrahedron Letters* 669.
9. Lunnon, M. W., MacMillan, J. and Phinney, B. O. (1977) *J. Chem. Soc. Perkin Trans.* 1, 2308.
10. Lewis, N. J. and MacMillan, J. (1980) *J. Chem. Soc. Perkin Trans.* 1, 1270.
11. Bohlmann, F., Jakupovic, J., King, R. M. and Robinson, H. (1980) *Phytochemistry* **19**, 863.
12. Beale, M. J., Bearder, J. R., MacMillan, J., Matsuo, A. and Phinney, B. O., (1983) *Phytochemistry* **22**, 875.
13. Bearder, J. R., MacMillan, J., Matsuo, A. and Phinney, B. O. (1979) *J. Chem. Soc. Chem. Commun.*, 649.
14. Bearder, J. R., MacMillan, J. and Phinney, B. O. (1975) *J. Chem. Soc. Perkin Trans.* 1, 721.
15. Beale, M. H., Bearder, J. R., Hedden, P., Graebe, J. E., and MacMillan, J. (1984) *Phytochemistry* **23**, 565.
16. Wada, K., Imai, T. and Yamashita, Y. (1981) *Agric. Biol. Chem.* **45**, 1833.
17. Yokota, T., Murofushi, N., Takahashi, N. and Tamura, S. (1971) *Agric. Biol. Chem.* **35**, 573.
18. Sweeley, C. C., Bentley, R., Makita, M. and Wells, M. M. (1963) *J. Am. Chem. Soc.* **85**, 2497.