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THE BIOSYNTHESIS OF KAURENOLIDE DITERPENOIDS BY *GIBBERELLA FUJIKUROI*

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Key Word Index—*Gibberella fujikuroi*; resuspension cultures; mutant B1-41a; kaurenoid biosynthesis; 7β-hydroxykaurenolide.

Abstract—The biosynthesis of 7β-hydroxy- and 7β,18-dihydroxy-kaurenolides from *ent*-kaur-16-en-19-oic acid has been investigated by incubating unlabelled and labelled putative intermediates with resuspension cultures of *Bibberella fujikuroi*. The results eliminate pathways which require the loss of oxygen from the 19-oic acid and indicate that the likely pathway is via *ent*-kaura-6,16-dien-19-oic acid and its *ent*-6α,7α-epoxide.

INTRODUCTION

The kaurenolides are diterpenoid lactones which occur in the fungus, *Gibberella fujikuroi* [1–5] and in higher plants [6–8] and which are biosynthetically related to the gibberellin (GA) plant hormones. Several papers dealing with the biosynthesis of the kaurenolides in cultures of *G. fujikuroi* have been published. By dilution analysis of 4-day old cultures, incubated with *ent*-[17-^{14}C]kaur-16-ene (**1**), Hanson *et al.* [9] found that 0.8% of the radioactivity was incorporated into 7β-hydroxykaurenolide (*ent*-6β,7α-dihydroxykaur-16-en-19-oic acid 19,6-lactone) (**4**) and 5.45% into 7β,18-dihydroxykaurenolide (*ent*-6β,7α, 18-trihydroxykaur-16-en-19-oic acid 19,6-lactone) (**5**). They also found that *ent*-7α-hydroxy-[17-^{14}C]kaur-16-en-19-oic acid (**3**) was incorporated, after 5 days incubation, into 7β-hydroxykaurenolide (**4**) and 7β, 18-dihydroxykaurenolide (**5**) to the extent of 0.03 and 0.44% respectively. Earlier Cross *et al.* [10] had shown that 7β-hydroxy-[17-^{14}C]kaurenolide (**4**) was incorporated into 7β,18-dihydroxykaurenolide (**5**) to the extent of 43%, a result later confirmed by Hanson and Sarah [11]. From these results the biosynthetic pathway: **1**→**2**→**3**→**4**→**5** has been suggested.

However Hedden and Graebe [12] have recently provided convincing evidence that *ent*-kaura-6,16-dienoic acid (**7**) is an intermediate between *ent*-kaur-16-en-19-oic acid (**2**), and 7β-hydroxykaurenolide (**4**) in cell-free preparations from *Cucurbita maxima* endosperm. By comparison of the incorporation of *ent*-[^{14}C]kaurenoic acid (**2**) and *ent*-7α-hydroxy-[^{14}C]kaurenoic acid (**3**) into 7β,12α-dihydroxykaurenolide (**6**), they also conclude that the kaurenolide (**6**) is biosynthesized solely from *ent*-kaurenoic acid (**2**) via *ent*-kaura-6,16-dienoic acid (**7**). The disclosure of these results, using the cell-free preparation from *C. maxima* endosperm, prompts us to publish our observations, relating to the biosynthesis of kaurenolides in *G. fujikuroi*. The results were obtained using resuspension cultures of the mutant B1-41a in which diterpene biosynthesis is blocked at the step preceding *ent*-kaurenoic acid (**2**). The preparation of new compounds used as substrates is described in the Results and Discussion. A preliminary account of these results has been presented orally [13].

RESULTS AND DISCUSSION

Some time ago [14] we observed that *ent*-kaur-6,16-dienoic acid (**7**) was converted into 7β-hydroxykaurenolide (**4**) by the mutant B1-41a. After a 20 hr incubation, GC/MS analysis of the MeTMSi derivatized extract from the medium indicated that 7β-hydroxykaurenolide (**4**) was the only major product. However, although little substrate remained, the yield was low, indicating that most of the substrate and/or its metabolites were in the mycelium. In repeating the experiment, therefore, methylated extracts of both medium and mycelium were examined. Unmetabolized substrate (**7**) and 7β-hydroxykaurenolide (**4**) were identified by GC/MS and quantified by comparison of the areas of the GLC peaks with those of standard injections. The results (Table 1) show that, after 0.5 hr, most of the unmetabolized substrate was in the mycelium. After 2 hr there was no substrate in the medium and little in the mycelium and the conversion to 7β-hydroxykaurenolide was 52%. *ent*-Kaura-6,16-dien-19-oic acid (**7**) is therefore rapidly and efficiently converted into 7β-hydroxykaurenolide (**4**).

Three possible ways in which the dienoic acid (**7**) could be transformed into 7β-hydroxykaurenolide (**4**) were considered (Scheme 1): (a) direct cyclization to kaurenolide (**8**), followed by 7β-hydroxylation; (b) epoxidation, followed by hydration of the 6β,7β-

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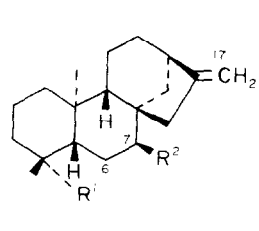

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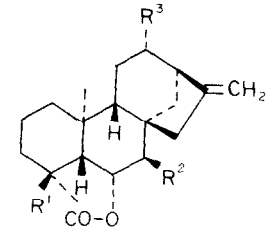
2 $R^1 = CO_2H, R^2 = H$
3 $R^1 = CO_2H, R^2 = OH$



4 $R^1 = Me, R^2 = OH, R^3 = H$
5 $R^1 = CH_2OH, R^2 = OH, R^3 = H$
6 $R^1 = Me, R^2 = R^3 = OH$

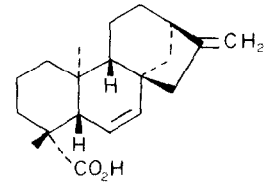


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Table 1. Analysis of the products obtained after incubation of *ent*-kaura-6,16-dien-19-oic acid (7) with mutant B1-41a*

Incubation time (hr)	Substrate (7)		7β-Hydroxykaurenolide (4)	
	Medium	Mycelium	Medium	Mycelium
0.5	5†	46.5	8.5	3.5
2.0	0	9.5	37.5	14.5

*GLC of methylated product on 2% SE-33 Column (170 × 0.2 cm) at 230°.

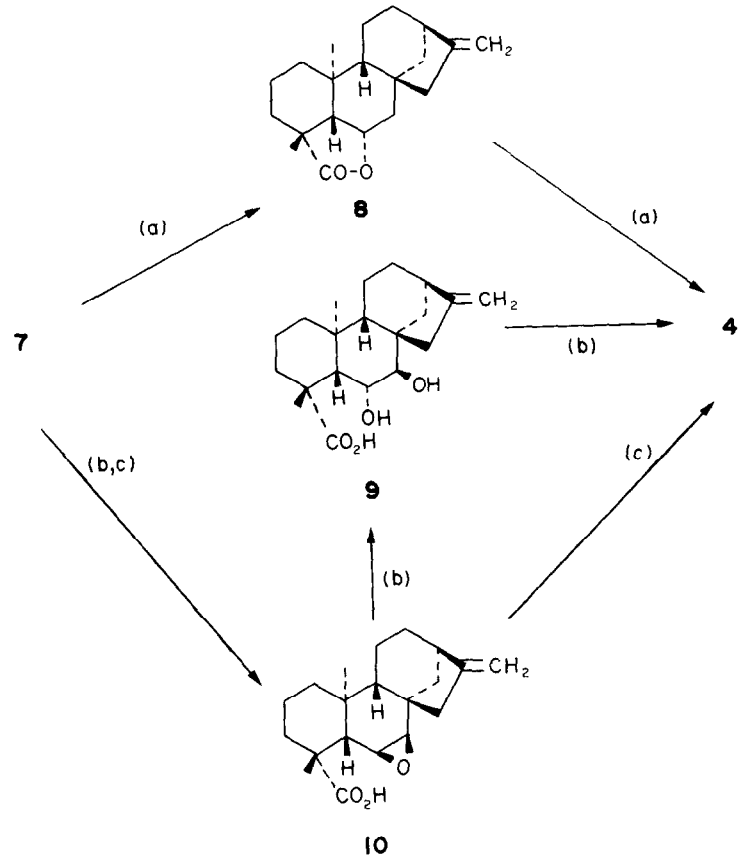
†Material recovered as a percentage of 7 added at the start of the incubation.

epoxide (**10**) to the diol (**9**) then lactonization; and (c) epoxidation followed direct attack of the 19-oic acid on the 6β, 7β-epoxide (**10**).

Route (a) had previously been tested by us [15] by incubating kaurenolide (**8**) with the mutant B1-41a. However, after 5 days, GC/MS of the MeTMSi derivatized extract from the culture filtrate indicated that the major product was the tri-acid (**11**) and this conclusion was supported by the ^1H NMR spectrum of the isolated metabolite. No evidence for the formation of 7β-hydroxy- or 7β,18-dihydroxykaurenolide (**4**) or (**5**) was obtained. Previously Hanson *et al.* [9] had found that kaurenolide (**8**) which had been added to an incubation of [17-^{14}C]kaurene with *G. fujikuroi* was recovered unlabelled. There is thus no evidence for the intermediacy of kaurenolide (**8**) in the biosynthesis of 7β-hydroxykaurenolide (**4**).

Route (b) (Scheme 1) had been previously tested by us [14]. Firstly, *ent*-6β, 7α-dihydroxykaur-16-en-19-oic acid (**9**) was incubated with the mutant B1-41a for 20 hr. The substrate was completely metabolized mainly to the di- and tri-acids (**12** and **11**) which were formed in the ratio of 1:4. No 7β-hydroxykaurenolide (**4**) was detected. Secondly, direct evidence that the kaurenolides (**4** and **5**) were not biosynthesized by lactonization of an *ent*-6β-alcohol such as **9** was obtained [16] by separately incubating unlabelled and [19-^{18}O]kaurenoic acid with resuspended cultures of B1-41a for 5 days at pH 3.0 and 4.5. In the neutral fraction from all four incubations, 7β,18-dihydroxykaurenolide (**5**) was identified as the bis-TMSi ether by GC/MS. Comparison of the intensities of the $[M-90]^+$ cluster, derived from labelled and unlabelled substrate, showed that the 7β,18-dihydroxykaurenolide (**5**), derived from *ent*-[19-^{18}O]kaurenoic acid containing 19.45 atoms ^{18}O per cent, contained 19.10 atoms ^{18}O per cent. The retention of ^{18}O-label excludes lactonization of an *ent*-6β-hydroxy-19-oic acid and also shows that the substrate (**2**) is not covalently bound, through the 19-oic acid, to an enzyme.

To test route (c) (Scheme 1), the epoxide (**10**) was prepared. However it was found to be readily converted into 7β-hydroxykaurenolide (**4**) when chroma-



Scheme 1. Possible biosynthetic routes from *ent*-kaura-6, 16-dien-19-oic acid (**7**) to 7β-hydroxykaurenolide (**4**).

tographed on Si gel. This non-enzymatic conversion also occurred rapidly in aqueous buffered solutions in the pH range 2.5–6.5 (Table 2) and was faster at the higher pH values indicating that attack on C-6 by the carboxylate anion is faster than that by the undissociated carboxyl. In view of these results it was not surprising that no significant difference in the rates of conversion of the epoxide (**10**) to 7β-hydroxykaurenolide (**4**) were observed for incubations of the epoxide (**10**) with live and boiled mycelium of the mutant B1-41a in citrate buffer at pH 2.9 (Table 3). Similar results were obtained for incubations in 0% ICI medium at pH 4.7. For reasons which are unknown conversion of the epoxide (**10**) was faster in the absence of boiled and live mycelium. It was not therefore possible to show directly that the epoxide (**10**) is an enzymatic intermediate between *ent*-kaur-6,16-dien-19-oic acid (**7**) and 7β-hydroxykaurenolide (**4**). However, the case for the intermediacy of the epoxide (**10**) is a strong one in view of the observed non-enzymatic conversion of **10** to **4**. The biosynthetic pathway (c) (Scheme 1) is therefore proposed.

Table 2. Conversion of the epoxide (**10**) to 7β-hydroxykaurenolide (**4**) in aqueous buffer

pH	Ratio epoxide–7β-hydroxykaurenolide* after 10 hr at 26°
2.51	1:2.87
3.30	1:3.57
4.30	1:18.2
5.10	1:80.6
6.63	1:261.5

*By GLC of methylated product on 2% SE-33 column (170 × 0.2 cm) at 230°.

ent-Kaur-6,16-dienoic acid (**7**) may be biosynthesized from *ent*-kaurenoic acid (**2**) either by direct dehydrogenation or by dehydration of *ent*-7α-hydroxykaurenoic acid (**3**). It is known that the *ent*-6α-hydrogen of *ent*-kaurenoids, although lost in the biosynthesis of GAs [17], is retained in the kaurenolides [18]. *ent*-7α-Hydroxy-[6α-^{2}H,15,17-^{3}H] kaurenoic acid was incubated with resuspension cultures of the mutant B1-41a. After 3 days the metabolites from the medium were seperated into acidic and neutral fractions. The acid fraction was analysed as the MeTMSi derivatives by GC/MS and GC/RC and shown to contain the normal metabolites, GA_9, GA_{25}, GA_{14}, GA_{13}, GA_1, GA_3, the di-acid (**12**) and the tri-acid (**11**) in tritiated form. The neutral metabolites were fractionated by preparative TLC and, with the aid of authentic markers, 7β-hydroxy- and 7β,18-dihydroxykaurenolides (**4** and **5**) were identified as the TMSi derivatives by GC/MS and shown by GC/RC of these derivatives to contain 0.017 and 0.087% of the radioactivity fed. The mass spectra of the TMSi derivatives showed a weak $[M]^+$

Table 3. Incubation of the epoxide (**10**) with *G. fujikuroi*, mutant B1-41a in citric acid–Na_2HPO_4 buffer at pH 2.9

		Peak area of epoxide (**10**)	Peak area of **4**	% of epoxide (**10**)	% of **4**
Substrate (**10**)		1098	—	100	—
7β-hydroxykaurenolide (**4**)		—	1298	—	100
Live fungus	1 hr	64	436	5.8	33.6
	2 hr	13	512	1.2	39.4
	3 hr	10	514	0.9	39.6
Boiled fungus	1 hr	181	352	16.5	27.1
	2 hr	71	563	6.5	43.4
	3 hr	34	582	3.1	44.8
No fungus	1 hr, 2 hr, 3 hr	—	~550	—	~42

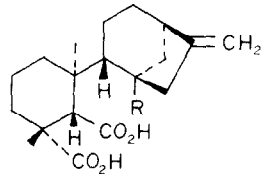
*From GLC of methylated product on 2% SE-33 column (170 × 0.2 cm) at 230°.

ion and a strong $[M - TMSiOH]^+$ ion from loss of the 7β-OTMSi group. Thus the presence or absence of deuterium could not be determined from these spectra. However the *t*-butyl dimethylsilyl (TBDMS) ethers [19] showed strong ions at $[M - C_4H_9]^+$ and $[M - C_6H_{13}]^+$ which did not contain deuterium. This result, showing that the kaurenolides **4** and **5** are not formed from *ent*-7β-hydroxykaurenoic acid (**3**), differs from previous conclusions by ourselves [20] and by Hanson *et al.* [9] but are in accord with the recent results of Hedden and Graebe for the *C. maxima* system [12]. Corroborative evidence for the non-intermediacy of *ent*-7α-hydroxykaurenoic acid (**3**) in the biosynthesis of fungal kaurenolides may also be inferred from the work of Bearder *et al.* [21]. These authors fed steviol (**13**) to cultures of the mutant B1-41a and obtained the 13-hydroxyanalogues (**14**) and (**15**) of *ent*-7α-hydroxykaurenoic acid (**3**) and 7β-hydroxykaurenolide (**4**). However, when *ent*-7α, 13-dihydroxykaurenoic acid (**14**) was re-incubated, no kaurenolides were detected. It would appear, therefore, that the dienoic acid (**7**) is formed by direct dehydrogenation of *ent*-kaurenoic acid (**2**) and since it has been shown [18] that the 6β- and 7α-hydrogens of *ent*-kaurene (**1**) are retained in 7β-hydroxykaurenolide (**4**), by *trans*-elimination of the 6α- and 7β-hydrogens.

In summary the pathway **1**→**2**→**7**→**10**→**4**→**5** is proposed for the biosynthesis of kaurenolides in cultures of *G. fujikuroi*. In view of the rapid metabolism of the dienoic acid (**7**) by cultures of the mutant B1-41a and of the instability of the corresponding 6β, 7β-epoxide (**10**), it is not surprising that these two postulated intermediates could not be detected in wild-type cultures of *G. fujikuroi*.

Preparation of substrates

Kaurenolide (**8**) was obtained by *n*-Bu_3SnH reduction of the thiobenzoate (**16**) of 7β-hydroxykaurenolide (**4**); it had previously been prepared from 7β-hydroxykaurenolide (**4**) by the published method [22] but, in our hands this route led to complications [15] which will be discussed in a separate paper [29]. *ent*-6β,7α-Dihy-



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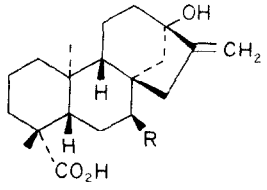
12 R = CHO



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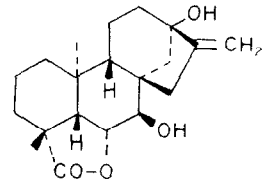
14 R = OH



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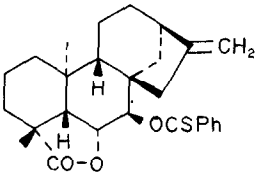
droxykaurenoic acid (**9**) was obtained from the kaurenolide (**4**) by treatment with aq. methanolic NaOH; the methyl ester has been described by Cross *et al.* [1]. *ent*-6β, 7β-Epoxykaurenoic acid (**17**) was prepared by treatment of the mesylate (**18**) with base and it was deoxygenated to *ent*-kaura-6, 16-dienoic acid (**7**) by the method of Cornforth *et al.* [23]. The dienoic acid acid (**7**) was also obtained by heating the toluene-*p*-sulphonate of *ent*-7α-hydroxy-kaurenoic acid (**3**) with collidine but it was most conveniently prepared by treatment of the mesylate (**18**) with sodium naphthalenide in THF. The dienoic acid (**7**) was methylated then treated with *m*-chloroperbenzoic acid to give the diepoxide (**21**) which was selectively deoxygenated with potassium selenocyanate [24, 25] to the monoepoxy ester (**22**). The latter was hydrolysed with NaSEt in HMPA immediately before use of the resultant epoxy-acid (**10**).

The β-stereochemistry of the epoxy-ester (**22**) was evident by comparison of the ^{1}H NMR spectrum with that of the 6α, 7α-epoxide (**17**). The C-20 protons in the β-epoxide occurred at higher field than in the α-epoxide (δ 0.83 vs 1.10). In both epoxides the 7-H signal was a doublet (J = 4 Hz) at *ca* 3.0. However the 6-H signal was a doublet (J = 4 Hz) at 3.15 in the α-epoxide (**17**) and a double-doublet (J = 2 and 4 Hz) at 3.86 in the β-epoxide (**22**). These data agree with those given by Hanson and Hawker [26] for the epoxides **23** and **24**.

ent-[19-^{18}O]Kaurenoic acid was prepared by refluxing the methyl ester of *ent*-kaurenoic acid (**2**), first in *t*-BuOH and then in diglyme, in the presence of 18-crown-6-ether, K and ^{18}O-enriched H_2O (72.9 atoms percent). The incorporation of ^{18}O into the acid (**2**), determined by GC/MS of the TMSi ether was only 19.45 atoms percent, suggesting that hydrolysis under these conditions occurred mainly by *O*-alkyl fission.

ent-7α-Hydroxy-[6α-^{2}H,15,17-^{3}H]kaurenoic acid was prepared from the mesylate (**18**) which was oxidized to the norketone (**19**). Protection of the ketone as the ethylene ketal (**20**), then treatment with sodium naphthalenide in $(MeOCH_2)_2$ gave the 6-ene (**25**). Treatment of the latter compound with deuterioborane generated externally from $NaBD_4$ and $BF_3.Et_2O$, followed by treatment of the product with toluene-*p*-sulphonic acid in Me_2CO gave the deuterated ketone (**26**). Hydrolysis of the methyl ester (**26**) with LiI in collidine and treatment of the resultant acid (**27**) with $Ph_3{}^+PC^3H_3Br^-$ and *t*-BuOK gave *ent*-7α-hydroxy[6α-^{2}H, 15, 17-^{3}H]kaurenoic acid with a sp. act. of 87 mCi/mmol and containing 62 atoms percent deuterium.



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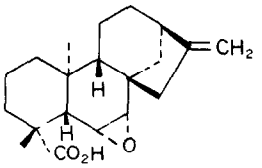

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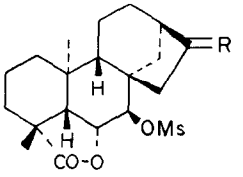

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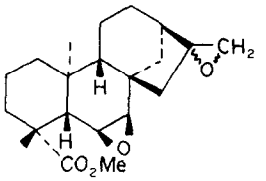
20 R = $-OCH_2CH_2O-$



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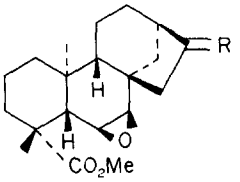

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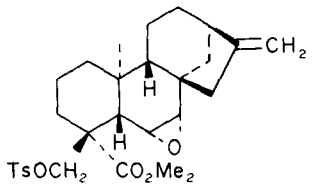

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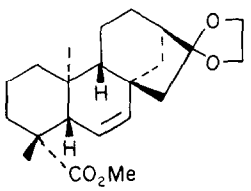

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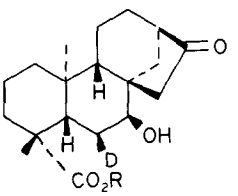

26 R = Me
27 R = H

EXPERIMENTAL

General experimental procedures. As described previously [27].

ent-6β,7α-*Dihydroxykaur*-16-*en*-19-*oic acid* (**9**) (*cf ref.* [1]). 7β-Hydroxykaurenolide (**4**) (500 mg) in EtOH (25 ml) was refluxed for 20 hr with 0.5 N NaOH (15 ml) to give the required acid (9), mp 235–237° (Found: $[M]^+$ 336.214. $C_{20}H_{30}O_4$ requires $[M]^+$ 336.214); IR ν_{max} cm^{-1}: 3350 *br*, 3070 *w*, 1690, 1655 and 870. The ester (CH_2N_2) crystallized from Me_2CO–petrol and had mp 228–231° (lit. [1], 228–231°) MS *m/z* (rel. int.) 384 $[M]^+$ (4), 330(15), 312(9), 298(15), 270(19), 137(45), and 109(100).

ent-6β-*Hydroxykaur*-16-*en*-19-*oic acid* 19, 6-*carbolactone* (*kaurenolide*) (**8**). 7β-Hydroxykaurenolide (**4**) (500 mg) in THF (5 ml) was treated with 5 ml $PC(Cl)=NMe_2Cl$ in CH_2Cl_2, prepared as in ref. [28]. After 24 hr at room temp., dry pyridine (1 ml) was added, followed by H_2S gas. After 10 min the soln was diluted with EtOAc, washed with H_2O and the yellow oil, recovered from the organic layer, was chromatographed on Si gel (80 g, 25 × 2.5 cm). Fractions eluted with petrol (200 ml), 5% EtOAc in petrol (250 ml) and 7.5% EtOAc in petrol (200 ml) were discarded. Elution with 10% EtOAc in petrol (850 ml) gave the yellow thiobenzoate (**16**) (570 mg), mp 216–219° (dec) (from Me_2CO) (Found: C, 73.8; H, 7.7; S, 7.2. $C_{27}H_{32}O_3S$ requires C, 74.3; H, 7.4; S, 7.3%). IR ν_{max} cm^{-1}: 1771, 1661, 1595, 1450, 1220, 886, 782, 690, ^1H NMR: δ 1.07 (*s*, 20-H_3), 1.3(*s*, 18-H_3) 1.95 (*d*, *J* = 7 Hz, 5-H), 4.96 (*t*, *J* = 7 Hz, 6-H), 4.9 (*br*, 17-H_2), 7.12 (*d*, *J* = 7 Hz, 7-H), 7.39 (*m*, 3 × ArH) and 8.17 (*m*, 2 × ArH); MS *m/z* (rel. int.): 436 $[M]^+$ (0.7), 403 (1.5), 298 (100), 283 (35), 270 (16), 255 (22), 137 (23), 121 (30), 109 (28) and 105 (31).

The thiobenzoate (**16**) (200 mg) in toluene (20 ml), containing 2,2'-azo-bis(2-methylpropionitrile) (5 mg) was added over 0.5 hr to refluxing toluene containing *n*Bu_3SnH (300 μl). After a further 0.5 hr, the toluene was removed under vacuum and the residual oil triturated with Me_2CO to give kaurenolide (**8**) (93 mg), mp 234–236° (with sublimation at 220°) (lit. [22], mp 204–205°); IR ν_{max} cm^{-1}: 1753, 1660 and 880; MS *m/z* (rel. int.): 300 $[M]^+$ (10), 285 (57), 257 (18), 256 (14), 241 (35), 239 (22), 229 (17), 211 (30), 123 (45) and 109 (100); ^1H NMR: δ 0.90 (*s*, 20-H_3), 1.26 (*s*, 18-H_3), 4.82 and 4.96 (each *br*, 6-H and 17-H_2).

ent-6β,7β-*Epoxykaur*-16-*en*-19-*oic acid* (**17**). The kaurenolide (**4**) (550 mg) was treated with $MeSO_2Cl$ (1.2 g) in pyridine (10 ml) for 2 days at room temp. The usual work-up gave the mesylate (**18**) (450 mg), mp 195–196° (from EtOAc–petrol) (Found: $[M]^+$ 384.181. $C_{21}H_{30}O_5S$ requires $[M]^+$ 384.181.); IR ν_{max} cm^{-1} 3040, 1785 and 1660; ^1H NMR: δ 0.92 (*s*, 20-H_3), 1.50 (*s*, 18-H_3), 1.85 (*d*, *J* = 7 Hz, 5-H), 3.16 (*s*, OSO_2Me), 4.80 (*t*, *J* = 7 Hz, 6-H), 4.88 and 5.02 (each *br*, 17-H_2), and 5.53 (*d*, *J* = 7 Hz, 7-H).

The mesylate (**18**) and KOH (1.5 g) in *t*-BuOH (40 ml) were refluxed for 5 hr. The cooled soln was added to 3 M HCl and the mixture extracted with EtOAc. The product, recovered from the EtOAc, was purified by prep. TLC on Si gel with EtOAc–petrol–AcOH (50:50:1) to give the required epoxide (**17**) as a gum (300 mg); IR ν_{max} cm^{-1}: 3400–2500 *br*, 1708 and 880; ^1H NMR: δ 1.10 (*s*, 20-H_3), 1.42 (*s*, 18-H_3), 3.00 (*d*, *J* = 4 Hz, 7-H), 3.65 (*d*, *J* = 4 Hz, 6-H), and 4.85 (*br*, 17-H_2). Treatment of **17** with CH_2N_2 gave the methyl ester, MS (rel. int.): *m/z* 330 $[M]^+$ (4), 315 (27), 312 (10), 271 (100), 121 (87), 105 (67) and 91 (87).

ent-*Kaura*-6,16-*dien*-19-*oic acid* (**7**). (a) *ent*-7β-Hydroxykaurenoic acid (**3**) (260 mg) in pyridine (2 ml) was treated with tosyl chloride (1.5 g) for 7 days. The normal work-up gave a foam which was chromatographed on Si gel column. The tosylate (293 mg), eluted with 40% EtOAc in petrol, was refluxed with collidine (10 ml) for 13 hr. The usual work-up gave a gum which was fractionated by prep. TLC on Si gel with EtOAc–petrol–AcOH (30:70:1). The fraction, recovered from the band at R_f 0.6, was crystallized from MeOH–H_2O to give the required dienoic acid (**7**) (112 mg) mp 136–137° (Found: 300.208. $C_{20}H_{28}O_2$ requires $[M]^+$ 300.209.) MS *m/z* (rel. int.): 300$[M]^+$ (87), 285 (27), 257 (27), 255 (52), 239 (59), 229 (100), 211 (65), 143 (59), and 91 (95); IR ν_{max} cm^{-1}: 2500–3700 *br*, 1697, 1655, 887 and 875; ^1H NMR: δ 0.90 (*s*, 20-H_3), 1.28 (*s*, 18-H_3), 2.22 (*br*, 15-H_2), 2.62 (*br*, 13-H), 4.82 (*br*, 17-H_2), 5.35 (*dd*, *J* = 10 and 4 Hz, 6-H), and 6.03 (*dd*, *J* = 10 and 1.5 Hz, 7-H). The methyl ester was prepared with CH_2N_2, MS *m/z* (rel. int.): 314 $[M]^+$ (62), 299 (27), 286 (26), 255 (62), 254 (75), and 239 (100).

(b) The mesylate (200 mg) (**18**) of the kaurenolide (**4**), prepared as described in the previous expt, was dissolved in THF (15 ml) and cooled to −63° under N_2 gas. Sodium naphthalenide (0.5 M in glyme) was added until the intense green colour persisted. After 0.5 hr stirring, the reaction was quenched with THF–H_2O. The THF was removed by evaporation under vacuum, H_2O was added, and the soln extracted with petrol to remove the naphthalene. The aq. soln was acidified to pH 3.0 with 2 MHCl and extracted with EtOAc. The oil, recovered from the EtOAc extract, was purified as in (a) to give the required dienoic acid (**7**) (55 mg).

(c) *ent*-6β, 7β-Epoxykaur-16-en-19-oic acid. (**17**) (312 mg) in AcOH (50 ml) and H_2O (0.35 ml) was stirred with NaOAc (1 g), NaI (500 mg) and Zn dust (600 mg). After 3 hr the mixture was filtered, diluted with H_2O and extracted with EtOAc. Purification of the recovered product by prep. TLC on Si gel with EtOAc–petrol–AcOH (40:60:1) gave the required dienoic acid (**7**) (208 mg).

Methyl ent-6α, 7α, 16, 17-*diepoxykauren*-19-*oate* (**21**). The dienoic acid (**7**) (80 mg) in MeOH was methylated (CH_2N_2) and the recovered methyl ester in $CHCl_3$ (20 ml) was treated overnight with *m*-chloroperbenzoic acid (300 mg). The $CHCl_3$ was evaporated under vacuum and the residue, in EtOAc, was washed with 5% aq. NaOH then H_2O. Evaporation and purification of the residue by prep. TLC on Si gel with EtOAc–petrol–AcOH (50:50:1) gave the diepoxide (**21**) (65 mg); ^1H NMR: δ 0.88 (*s*, 20-H_3), 1.39 (*s*, 18-H_3), 2.91 (*q*, *J* = 5 Hz, 17-H_2), 3.07 (*d*, *J* = 4 Hz, 7-H), 3.72 (*s*, CO_2Me) and 3.91 (*dd*, *J* = 2 and 4 Hz, 6-H); MS *m/z* (rel. int.): 346 $[M]^+$ (9), 331 (11), 318 (26), 314 (12), 287 (48), 121 (46), and 109 (100).

Methyl ent-6α, 7α-*epoxykaur*-16-*en*-19-*oate* (**22**). The diepoxide (**21**) (230 mg) in MeOH (30 ml) was treated with KSeCN (1.25 g) under reflux for 5 hr. The solvent was removed *in vacuo* and the residue was partitioned between EtOAc and H_2O. Evaporation of the EtOAc and prep. TLC on Si gel with EtOAc–petrol–AcOH (50:50:1) gave the *ent*-6α,7α-epoxide (**22**) (112 mg) mp 111–112° (from Me_2CO) (Found: C, 75.9; H, 9.4. $C_{21}H_{30}O_3$ requires C, 76.3; H, 9.2%.) IR ν_{max} cm^{-1} 1730, 1660, 880: MS (rel. int.): *m/z* 330 $[M]^+$ (29), 315 (7), 312 (13), 299 (12), 270 (48), and 147 (100); ^1H NMR; δ 4.87 (*br*, 17-H_2), 3.86 (*dd*, *J* = 4 and 2 Hz, 6-H), 3.69 (*s*, CO_2Me), 2.94 (*d*, *J* = 4 Hz, 7-H), 1.37 (*s*, 18-H_3), and 0.83 (*s*, 20-H_3).

ent-6α,7α-*Epoxykaur*-16-*en*-19-*oic acid* (**10**). To NaH (1.8 g of 50% dispersion in oil, washed with petrol) in dry HMPA (20 ml) was added EtSH (1.0 ml) dropwise whilst stirring under N_2 gas. After stirring for 1 hr, the solids were allowed to settle and the supernatant soln (15 ml) added to the epoxy-ester (**22**) (25 mg). After 2.5 hr the reaction mix-

ture was diluted with H_2O and washed with EtOAc. Careful acidification of the aq. layer with conc. HCl to pH 4 and recovery in EtOAc (H_2O backwash) gave the crude epoxy-acid (**10**). Methylation of an aliquot with CH_2N_2 gave a single GLC peak identical (GC/MS) with the starting methyl ester.

Attempted purification by prep. TLC on Si gel with EtOAc–petrol (1:1) gave a single band at R_f 0.5 which when eluted was shown to be identical (GLC and ^{1}H NMR) with 7β-hydroxykaurenolide (**4**).

Stability of the epoxy-acid (**10**) *in buffered media.* A range of citric acid–Na_2HPO_4 buffers were prepared. To each was added glucose (16 g/200 ml buffer), $MgSO_4 \cdot 7H_2O$ (200 mg/200 ml buffer) and trace elements soln. (400 μl/200 ml buffer). In this way solns of pH 2.51, 3.30, 4.30, 5.10 and 6.63 were obtained. To each soln (50 ml) was added *ca* 1 mg freshly prepared epoxy-acid (**10**) in Me_2CO (100 μl). The solns were shaken at 26° on a rotary shaker. After 10 hr the solns were extracted with EtOAc and the washed (H_2O) extracts immediately treated with CH_2N_2 and analysed by GLC on a 2% SE–33 column at 230°. The results are described in the Results and Discussion.

ent-[19-^{18}O]*Kaur*-16-*en*-19-*oic acid.* K metal (13 mg) in *t*-BuOH (0.5 ml) containing 18-crown-6-ether (crystal) and H_2O (72.9 atom per cent ^{18}O, 10 μl) was added to the methyl ester of *ent*-kaurenoic acid (**2**) (10 mg) in *t*-BuOH (1.5 ml). The soln was refluxed for 1 hr and evaporated by a stream of N_2 gas. Dry diglyme (2 ml) was added, the soln refluxed for 1 hr and evaporated as before. KH_2PO_4 buffer (1 M, 4 ml) was added and the product recovered in EtOAc to give *ent*-kaurenoic acid (**2**) (8.2 mg), one peak by GC/MS as the TMSi ester on 2% SE-33 (170 × 0.2 cm) temp. programmed from 200° at 2° min with an He flow rate, 30 ml/min.

The average $[M]^+$ values were 374.7235 for the ^{18}O enriched compound and 374.3348 for the unlabelled standard showing the incorporation of 19.454% atoms ^{18}O percent.

7β-*Mesyloxykaurenolide norketone* (**19**). 7β-Mesyloxykaurenolide (**18**) (200 mg) in THF (20 ml) was stirred with a small crystal of OsO_4 for 10 min when H_2O (10 ml) and sodium metaperiodate (530 mg) were added. After 24 hr the mixture was concd *in vacuo*, diluted with H_2O and extracted with EtOAc. On evaporation the EtOAc layer gave a brown oil which crystallized from EtOAc–petrol giving 7β-mesyloxykaurenolide norketone (**19**) (176 mg) mp 216–218°. (Found: $[M]^+$ 396.161. $C_{20}H_{28}O_6S$ requires M, 396.161.) IR ν_{max} cm^{-1}: 1778, 1745 and 939; ^{1}H NMR δ 0.99 (*s*, 20-H$_3$), 1.32 (*s*, 18-H$_3$), 1.82 (*d*, J = 7 Hz, 5-H), 3.22 (*s*, OSO_2Me), 4.79 (*t*, J = 7 Hz, 6-H), and 5.34 (*d*, J = 7Hz, 7-H); MS *m/z* (rel. int.): $[M]^+$ (8), 352 (2), 300 (100), 272 (27), 257 (36), 241 (54), 137 (35), and 109 (68).

7β-*Mesyloxy*-16-*ethylenedioxy*-17-*norkaurenolide* (**20**). 7β-Mesyloxykaurenolide norketone (**19**) (140 mg) in C_6H_6 (10 ml) containing $(CH_2OH)_2$ (1 ml) and *p*-$MeC_6H_4SO_3H$ (22 mg) was refluxed for 24 hr in a Dean and Stark apparatus. The C_6H_6 was evaporated *in vacuo* and the residue was neutralized with NH_4OH and extracted with EtOAc. Recovery from the EtOAc layer gave 7β-mesyloxy-16-ethylenedioxy-17-norkaurenolide (**20**) (90 mg) mp 214.5–216° (from EtOAc–petrol). (Found: C, 60.11; H, 7.23; S, 7.35. $C_{22}H_{30}O_7S$ requires C, 59.88; H, 7.32; S, 7.31.) IR ν_{max} cm^{-1} 1781; ^{1}H NMR: δ 0.90 (*s*, 20-H$_3$), 1.30 (*s*, 18-H$_3$), 3.18 (*s*, OSO_2Me), 3.85 (*m*, OCH_2CH_2O), 4.75 (*t*, J = 7Hz, 7-H), and 5.24 (*d*, J = 7 Hz, 6-H); MS *m/z* (rel. int.). 438 $[M]^+$ (13), 423 (3), 359 (48), 344 (21), 213 (26), 166 (50), 112 (15) and 86 (100).

Methyl 16, 16-*ethylenedioxy*-17-*norkaur*-6-*enoate* (**25**). A 0.3 M soln of sodium naphthalenide in dry $(MeOCH_2)_2$ was added dropwise to a stirred soln of 7β-mesyloxy-16-ethylenedioxy-17-norkaurenolide (**20**) (250 mg) in dry $(MeOCH_2)_2$ (30 ml) under N_2 until the green colour persisted. The reaction was then quenched with H_2O and concd *in vacuo*. More H_2O was added and the alkaline soln was extracted with petrol. The petrol which contained mostly naphthalene was discarded. The aq. layer was cautiously acidified with 2 M HCl to pH3 and extracted with Et_2O. Evaporation of the Et_2O gave an oil (165 mg) which was methylated with CH_2N_2. Analysis by GC/MS showed this to be a mixture (85:15) of the required olefin (**25**) and the corresponding 16-ketone. Re-ketalization of the mixture as above gave, prep. TLC (30% EtOAc in petrol), methyl 16-ethylenedioxy-17-norkaur-6-en-19-oate (**25**) (48 mg); mp 114–116° (from aq. EtOH) (lit. [26], 115–116°); MS *m/z* (rel. int.): 360 $[M]^+$ (22), 301 (12), 274 (49), 214 (40), 199 (33), 145 (50), 113 (76), and 87 (100).

Methyl ent-7α-*hydroxy*-17-*nor*-16-*oxo*-6α-$^2H_1$-*kauran*-19-*oate* (**26**). Methyl 16, 16-ethylenedioxykaur-6-en-19-oate (**25**) (130 mg) in dry THF (8 ml) was treated with deuterioborane generated externally from a solution of $NaBD_4$ (110 mg) in $(MeOCH_2CH_2)_2O$ (10 ml) treated with BF_3Et_2O–$(MeOCH_2CH_2)_2O$ (1:5) (added dropwise over 10 min). After 3 hr, 2N NaOH (2 ml) and H_2O_2 (0.5 ml) were added and the mixture stirred for 0.5 hr. The THF was evaporated and the mixture diluted with H_2O and aq. KH_2PO_4. Extraction with EtOAc gave a white solid (150 mg). Analysis by GC/MS showed this to contain some starting material but mainly the required product as its 16-ethylene ketal; MS (rel. int.): *m/z* 451 $[M]^+$ (20), 450 (7), 174 (100), 113 (18), 87 (18), and 73 (35). The crude product was dissolved in Me_2CO (3 ml) containing *p*-$MeC_6H_4SO_3H$ (10 mg). After stirring overnight the solvent was evaporated, H_2O was added and the mixture was extracted with EtOAc. Prep. TLC of the EtOAc extract on HF-Si gel with 40% EtOAc in petrol yielded (R_f 0.2) the required norketone (**26**) (47 mg) identical with authentic material by MS (apart from the expected isotope shift); MS *m/z* (rel. int.): 407 $[M]^+$ (5), 406 (3), 392 (9), 391 (4), 317 (23), 275 (26), 274 (15), 258 (38), 257 (29), and 73 (100).

ent-7α-*Hydroxy*-[6α-^{2}H;15,17-^{3}H] *kaur*-16-*en*-19-*oic acid.* (a) The norketone (**26**) (45 mg) and LiI (220 mg), in dry collidine (2.5 ml) were heated under reflux for 1 hr. The collidine was evaporated and the residue was acidified then extracted with EtOAc. Prep. TLC of the EtOAc extract on HF-Si gel in EtOAc–petrol–HOAc (40:60:1) (2 developments) yielded (R_f 0.5) the required acid (**27**) (30 mg) which was used directly in the following Wittig reaction.

(b) Methyltriphenylphosphonium bromide (180 mg) was dissolved in a mixture of MeCN (1.5 ml), Et_3NH (290 μl) and tritiated H_2O (40 μl; 5 Ci/ml) and the soln was heated at 55° for 3 hr then left overnight at room temp. The solvent was then evaporated under a stream of N_2 and the residue dried under vacuum at 100° over P_2O_5.

The tritiated phosphonium salt (176 mg) was dissolved in THF (2 ml) and treated with a soln (0.75 ml) of *t*-BuOK in THF (119 mg/ml) under N_2 with stirring. After 1 hr the norketone (**27**) (22 mg), in THF (0.5 ml), was added and stirring was continued overnight. The reaction was then quenched with Me_2CO (0.3 ml) and the solvent was removed under a stream of N_2. H_2O was added and the mixture was cautiously acidified to pH 2 with 2 M HCl. Extraction with EtOAc and recovery of the organic phase gave a gum (130 mg) which was chromatographed on HF-Si gel plates in EtOAc–petrol–AcOH (50:50:1). The band at R_f 0.5 gave *ent*-7α-hydroxy-[6α-$^2H_1$;15,17-$^3H_2$]kaur-16-en-19-oic acid (13 mg); mp 250–252° (from Me_2CO–petrol), crystallized to a

constant sp. act. of 87 mCi/mmole and containing 62 atoms percent deuterium.

Incubations with Gibberella fujikuroi, *mutant B1-41a.* Shake cultures (50 ml) of the mutant in 40% N ICI medium were grown for 3–4 days as previously described [20]. The pigmented mycelium was collected by filtration and resuspended in flasks containing 50 ml of the specified medium. Substrates (1–10 mg/50 ml resuspension medium) were added in Me_2CO (100 μl). After the specified time, the culture filtrate was adjusted to pH 8–10 with 2 M KOH and extracted with EtOAc to give a neutral–basic fraction A. The pH was then adjusted to 3.0 and extracted with EtOAc to give an acid extract B. The mycelium was extracted with MeOH to give extract C. On some occasions, the culture filtrate was extracted at pH 3.0 only to give fraction A + B.

Incubation of ent-*kaura*-6,16-*dien*-19-*oic acid* (**7**). The substrate (2 mg) was incubated with resuspended mycelium in 0% N ICI medium (50 ml) in two separate expts. One expt was worked-up after 0.5 hr and the other after 2 hr. Fractions (A + B) and C from each expt were treated with CH_2N_2 in Et_2O and analysed by GLC on 2% OV-210 and 2% SE-33 packed columns. The results are shown in Table 1.

Incubation of kaurenolide (**8**). The substrate (10 mg) was incubated for 5 days with mycelium resuspended in 0% N ICI medium (50 ml) at the natural pH 4.5. No 7β-hydroxy or 7β, 18-dihydroxykaurenolides of (**4** and **5**) were detected by GLC and GC/MS of the underivatized fraction A + B. The methylated fraction A + B showed a single large peak, with unchanged retention time after trimethylsilylation, which was tentively identified, by GC/MS and ^1H NMR of the total product (42 mg) from a larger scale incubation of 100 mg substrate and 450 ml resuspension culture, as the tri-acid (**11**).

Incubation of ent-6β,7α-*dihydroxykaur*-16-*en*-19-*oic acid* (**9**). The substrate (1 mg) was incubated with resuspended mycelium in 0% N ICI medium (50 ml) for 20 hr at pH 3.5. No kaurenolides were detected by GC/MS in fraction A + B. The di-acid (**12**) and tri-acid (**11**) were identified in fractions A + B as the methyl esters by GC/MS on a 2% QF-1 column (5 feet × 1/8 inches) at 2.5° with a He flow-rate of 30 ml/min.

Incubation of ent-[19-^{18}O]*kaur*-16-*en*-19-*oic acid.* The substrate (2 mg) (19.45 atoms ^{18}O percent) was incubated for 5 days in 0% N ICI medium (50 ml) both at the natural pH 4.5 and adjusted to pH 3.0 with 2 M HCl. Control incubations of unlabelled substrate (2 mg) were also performed at each pH value. Fractions B from all four expts were derivatized by methylation (CH_2N_2) then trimethylsilylation with TMCS–HMDS–pyridine (2:1:1) and the MeTMSi derivatives were analysed by GC/MS on 2% SP 2100 at 180° for 5 min. and then programmed at 3° with a He flow-rate of 30/ml min.

7β, 18-Dihydroxykaurenolide (**5**) was identified in all four expts. The average $[M-90]^+$ values were 386.8819 and 386.8833 a.m.u. from the ^{18}O-enriched substrate at pH 4.5 and pH 3.0 respectively and 386.4999 and 386.5013 a.m.u. from unlabelled substrate at pH 4.5 and pH 3.0 respectively. The ^{18}O contents of 7β,18-dihydroxykaurenolide (**5**) were therefore 19.099 atoms percent at the natural pH and 19.103 atoms percent at pH 3.

Incubation of ent-6α,7α-*epoxykaur*-16-*en*-19-*oic acid* (**10**). The substrate (1 mg) was incubated separately with (a) citrate–phosphate buffered medium prepared as above (pH 2.9), (b) resuspended mycelium in citrate–phosphate buffered medium and (c) boiled resuspended mycelium in citrate–phosphate buffered medium, for periods of 1, 2 and 3 hr. Similar incubations were performed in (a) 0% N ICI medium [20], (b) resuspended mycelium in 0% N ICI medium. and (c) boiled resuspended mycelium in 0% N ICI medium.

Fractions A + B were treated with CH_2N_2 in Et_2O and analysed by GLC and GC/MS on a 2% SE-33 at 230°. The results are shown in Table 3.

Incubation with ent-7α-*hydroxy*-[6α-^{2}H; 15, 17-^{3}H]*kaur*-16-*en*-19-*oic acid.* The substrate (2 mg) (469×10^6 dpm) was incubated with resuspended mycelium in 0% N ICI medium [20] for 3 days at the natural pH 4.5. Fraction A was purified by prep. TLC on Si gel with EtOAc–petrol (9:11) and the R_f zones corresponding to marker spots of 7β-hydroxykaurenolide (**4**) at R_f 0.7–0.9 (0.75×10^6 dpm) and of 7, 18-dihydroxykaurenolide (**5**) at R_f 0.2–0.4 (5.76×10^6 dpm) were eluted with Me_2CO. Aliquots of each extract were analysed as the MeTMSi and MeTBDMSi [19] derivatives by GC/MS on 2% OV-210 temp. programmed from 185° at 3°/min. Analysis of the MeTMSi derivatives by GC-RC on 2% OV-210 gave incorporation of 0.017% (0.081×10^6 dpm) for 7β-hydroxykaurenolide (**4**) and 0.087% (0.41×10^6 dpm) for 7β,18-dihydroxykaurenolide (**5**). Fraction B was analysed by GC/MS and GC-RC as its MeTMSi derivative on 2% OV-210 as above.

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