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METABOLIC TRANSFORMATIONS OF SOME *ENT*-KAURENES IN *GIBBERELLA FUJIKUROI*

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Key Word Index—*Gibberella fujikuroi*, fungus; gibberellin biosynthesis, *ent*-kaurene metabolism; gibberellic acid, *ent*-3β-hydroxykaur-16-en-19-yl succinate

Abstract—The conversion of *ent*-kaur-16-enes to gibberellic acid in *Gibberella fujikuroi* is blocked by A-ring modifications Thus *ent*-3β-hydroxykaur-16-en-19-yl succinate gives good conversion (46%) to the 7β-hydroxy derivative * Under the same conditions the 3β-epimer gives 7β- or 6α-hydroxylation and the former occurs for the 3-oxo analogue The succinoyloxy function acts as a less efficient block and *ent*-kaur-16-en-19-yl succinate is converted to 7β-hydroxy and 6β,7β-dihydroxy derivatives along with gibberellic acid. Hydrolysis of the succinate block of the metabolites provides the 7β,19-diol and 6β,7β,19-triol. Of this pair only the former was effectively metabolized to gibberellic acid in *G fujikuroi*

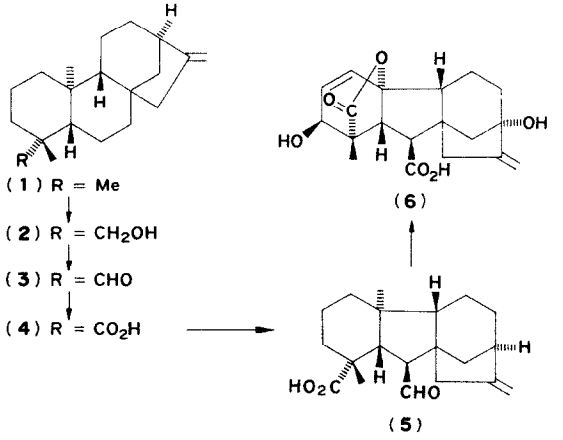
INTRODUCTION

AT THE outset of the work to be described, the biosynthesis of gibberellic acid (**6**) had been indicated to involve metabolism of *ent*-kaur-16-ene* (**1**) as in Scheme 1. Thus the hydrocarbon (**1**), the alcohol (**2**), aldehyde (**3**) and acid (**4**) were shown[4,5] to be related sequentially in *Echinocystis macrocarpa* endosperm homogenate and they were each [4–8] converted into gibberellic acid (**6**) by *Gibberella fujikuroi*. In addition Cross[9] had shown that the gibberellin aldehyde (**5**) was probably a key intermediate. Despite the fact that ring contraction appeared to require oxygenation of C-6 and C-7[10] no B-ring functionalized kaurenes had been shown to be intermediates although a number had been tested.[11] Our approach to

* Rowe's systematic nomenclature proposals[1] for the diterpenes, which have been widely accepted[2,3] describe (−)-kaurene and the derived gibberellins as derivatives of *ent*-kaurane and *ent*-gibberellane. Confusion has arisen in the specification of the configuration of substituents in relation to structural formulae Thus the systematic name *ent*-7α-hydroxykaur-16-en-19-oic acid has been applied[3] to **23**b whereas this is commonly referred to as 7β-hydroxykaurenoic acid. The confusion in the description of the C-7 configuration is representative of many similar problems which can be avoided by adopting inverted formulae as in **23**a The recent IUPAC steroid rules ((1972) *Pure Appl Chem* **31**, 285) appear to preclude this practice and so we are adopting the procedure[3] in which substituent configurations are related directly to structures (i e *ent*-7α = 7β) Thus structural features will be described so that α and β refer directly to the structures shown On the other hand, the use of the operator *ent* is retained for the systematic names of compounds

[1] ROWE, J W (1968) *The Common and Systematic Nomenclature of Cyclic Diterpenes* Third Revision Forest Products Laboratory, U S Dept of Agriculture, Madison, Wisconsin, U S A
[2] LANG, A (1970) *Ann Rev Plant Physiol* **21**, 537
[3] HARRISON, D. M and MACMILLAN, J (1971) *J Chem Soc.* **C**, 631
[4] GRAEBE, J. E, DENNIS, D T, UPPER, C D. and WEST, C. A (1965) *J Biol Chem* **240**, 1847
[5] DENNIS, D T and WEST, C A (1967) *J. Biol Chem* **242**, 3293
[6] CROSS, B E, GALT, R H B and HANSON, J. R (1964) *J. Chem. Soc* 295
[7] GALT, R. H B (1965) *J. Chem Soc.* 3143
[8] GEISSMAN, T A, VERBISCAR, A J, PHINNEY, B O and CRAGG, G (1966) *Phytochemistry* **5**, 933; (1967) **6**, 807.
[9] CROSS, B. E., NORTON, K and STEWART, J C (1968) *J Chem Soc* **C**, 1054
[10] BIRCH, A J, RICKARDS, R W, SMITH, H, HARRIS, A and WHALLEY, W B (1959) *Tetrahedron* **7**, 241
[11] CROSS, B E, GALT, R H B and NORTON, K (1968) *Tetrahedron* **24**, 231

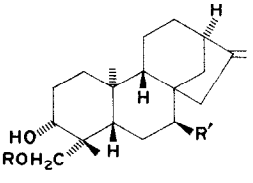
finding these intermediates was based on the expectation that if the mould could be induced to metabolize an *ent*-kaurene substituted to preclude formation of gibberellic acid the metabolism would be blocked along the pathway.



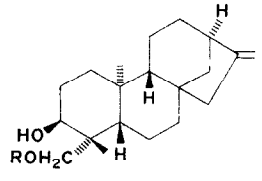
SCHEME 1

RESULTS AND DISCUSSION

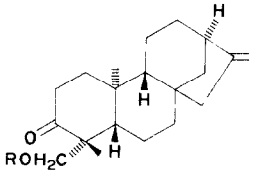
The first stage in the work was to screen a group of natural *ent*-kaurenes to establish if they were efficiently metabolized by *G fujikuroi*. The best result was obtained with the hydroxy succinate (**7**) readily available from *Goodenia strophiolata*,[12] the C-3 oxygenation in **7** is epimeric with the gibberellins and could provide an effective block. On the other hand if the succinate ester was retained it could be expected to block oxidation of C-19 to carboxyl In practice metabolism of **7** gave a major product which retained the succinate residue. Purification was most readily achieved by treatment of the unfractionated acidic fraction with base, which gave a triol (**8**) in 46% yield The IR spectrum showed retention



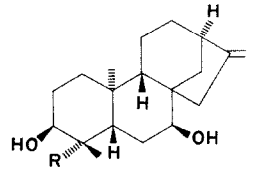
(**7**) R = $COCH_2CH_2CO_2H$; R′=H
(**8**) R = H; R′=OH
(**9**) R = COC_6H_5; R′= OH



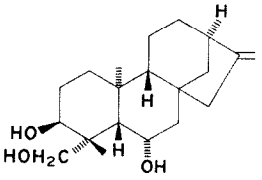
(**12**) R = H
(**13**) R = $COCH_2CH_2CO_2H$



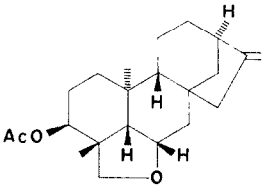
(**14**) R = $COCH_2CH_2CO_2Me$
(**15**) R = $COCH_2CH_2CO_2H$



(**16**) R = H
(**17**) R = COC_6H_5



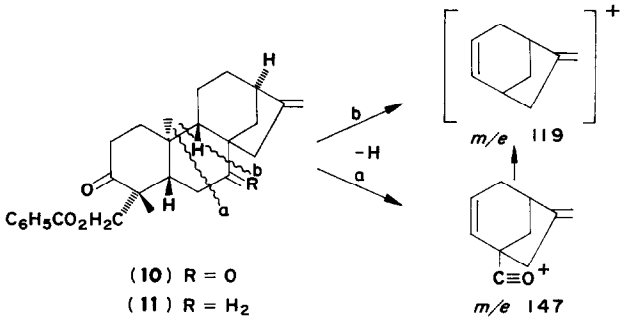
(**18**)



(**19**)

[12] MIDDLETON, E J and JEFFRIES P R (1968) *Australian J Chem* **21**, 2349

of the vinyl group and a broad absorption for hydroxyl. The NMR spectrum had the features of the parent diol together with a multiplet expected for the methine proton of another secondary alcohol. This multiplet was clearly resolved in the mono-benzoylation product (**9**) appearing at δ 3·69 with $W_{\frac{1}{2}}$ = 6 Hz suggesting an axial hydroxyl group. Oxidation of the mononobenzoate (**9**) gave a diketobenzoate (**10**) which did not show the enolate formation in base due to an α- or β-diketone thus excluding C-1 and C-2 for the new hydroxyl group. The presence of unperturbed C-13 H and C-17 H_2 resonances in **10** was inconsistent with oxygenation of C-12,[13] C-14[14] or C-15.[15] In addition shifts for the C-4 and C-10 Me groups in the triol and diketobenzoate were incompatible[16] with oxygenation at C-6. Although the deshielding of C-10 Me (0·23 ppm) in the diketobenzoate (**10**) in comparison with (**11**) is expected for a 7-ketone, it need not exclude C-11. However, the elaboration of 7-hydroxykaurenolide and oxygenated B-ring kaurenoid metabolites by the mould, along with the exclusion of hydroxylation at C-6, strongly suggested C-7 as the location of the metabolic hydroxyl group; in this case the NMR pattern for the C-7 H requires the hydroxyl to be axial. Confirmation of this view followed from the MS of the diketobenzoate which shows an intense peak at *m/e* 147 corresponding to cleavage of C-9, C-11 and C-6, C-7 as in Scheme 1. The origin of a strong peak at *m/e* 119 is also evident. Subsequently we found these fragmentations to be dominant processes in 7-oxokaurenes.



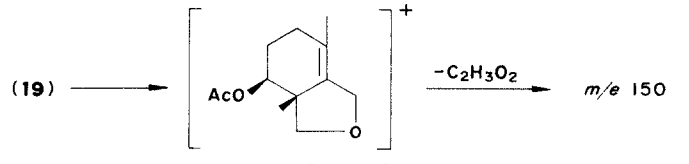
(**10**) R = O
(**11**) R = H_2

SCHEME 2

It was evident that our aim in securing efficient one-step metabolism of an *ent*-kaur-16-ene in *G. fujikuroi* had been achieved and we next looked to the effects of minor alterations to the blocking groups in the hope of advancing the metabolic sequence a further stage. Replacement of the 3α-hydroxyl group in **7** by the 3β-hydroxyl which occurs commonly in natural gibberellins was an obvious development. Earlier work had shown that the required diol (**12**) was formed together with the C-3 epimer by Ponndorf reduction of the 3-ketosuccinate (**14**).[17] A sample of diol obtained by this route was partially esterified with succinic anhydride and the succinate (**13**) obtained exposed to cultures of *G. fujikuroi*. After 7 days the acidic products were saponified and the neutral product separated to give the triols **16** and **18** in 38 and 11% yield respectively. Comparison of the spectral data for **16** with the 3α,7β,19-triol (**8**) strongly indicated that the new triol was also 7β-hydroxylated. In the benzoylation product (**17**) NMR signals at δ 3·92 and 3·64 ($W_{\frac{1}{2}}$ = 4·5 and 5·0 Hz)

[13] JEFFERIES, P R and RETALLACK, R W (1968) *Australian J Chem* **21**, 2085
[14] YOSHIKOSHI, A, KITADANI, M and KITAHARA, Y (1967) *Tetrahedron* **23**, 1175
[15] CANON, J R, CHOW, P W, JEFFERIES, P R and MEEHAN, G V (1966) *Australian J Chem.* **19**, 861
[16] GHISALBERTI, E L and JEFFERIES, P R (1968) *Australian J Chem* **21**, 439
[17] GHISALBERTI, E L, JEFFERIES, P R and MIDDLETON, E J (1969) *Australian J Chem.* **22**, 455

were well separated and were assigned to the equatorial protons at C-3 and C-7 respectively. Oxidation of the benzoylation product with Jones' reagent gave the diketobenzoate (**10**) previously obtained from the monobenzoate (**9**) thus confirming C-7 as the site of the metabolic hydroxyl group in **16**. The minor triol metabolite (**18**) also showed spectra for the kaurene nucleus substituted by two secondary and one primary hydroxyl group. Comparison of the methyl shifts for the triol and the parent 3β,19-diol (**12**) in C_5D_5N showed a downfield shift for one methyl group in the triol (29 Hz). Shifts of this magnitude could result only from hydroxylation at C-6,[16] C-2 (axial)[17] or possibly C-14;[14] a much larger value[13] is given for C-12 axial hydroxylation. When the triol was treated briefly with tosyl chloride–pyridine and then strong base a cyclic ether was obtained. The ether was characterized as the acetate (**19**) which showed a coupling constant for the C-19 H_2 (8 Hz) which corresponds well with values reported for five membered rings.[18] In principle an ether could be derived from a C-2 or C-6 axial hydroxyl group but C-2 can be excluded since the ether is not identical with **20** which we had available as a minor component of the base catalysed isomerization of *ent*-2α,3α-epoxykaur-16-en-19-ol[19] As expected for the acetate (**19**) the resonance for the 3 H requires a flanking methylene, but the $W_{\frac{1}{2}}$ for the signal (16 Hz) is not acceptable for the chair conformation of the A-ring On the other hand a twisted boat conformation greatly reduces steric compression on the α face and presumably accounts for the appearance of this signal The origin of the ether invites brief comment Tosylation of the triol (**18**) will certainly result in very much faster esterification of the primary alcohol than either secondary grouping. The product might in principle undergo fragmentation on treatment with base as we have shown for the 6-desoxy analogue.[17] However no evidence for products resulting from this process was obtained and evidently displacement of tosylate by the 6-hydroxyl group is a much faster process Strong support for the ether structure (**19**) is available from the MS. Thus the acetate shows intense peaks at *m/e* 209 (base peak) and *m/e* 150 which can be expected to arise as in Scheme 2



SCHEME 3

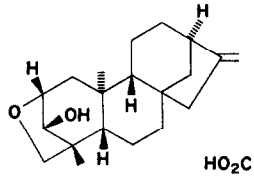
We then examined metabolism of the 3-keto succinate (**14**).[17] However the primary metabolites proved to be unstable and so the acidic fraction from the culture filtrates was reduced with $LiAlH_4$. This allowed isolation of a small quantity of the 3α,7β,19-triol (**8**).

The metabolic transformations described above together with the oxygenation patterns of the normal kaurenoid metabolites of *G. fujikuroi* strongly suggested that 7β-hydroxylation was the first step in conversion of the *ent*-kaurenoic acid (**4**) to gibberellins A test of this proposal could be made by removing 3-oxygenation from either the triol **8** or **16** since the derived 7β,19-diol could then be assessed directly as an intermediate in gibberellin biosynthesis. Other work has shown that the mould appears to have a general facility to

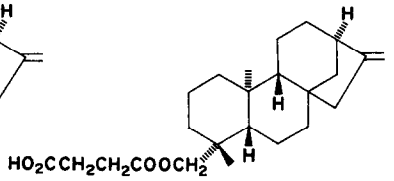
[18] COOKSON, R. C., CRABB, T. A., FRANKEL, I. L. and HUDEC, I. (1966). *Tetrahedron. Suppl.* 7, 355.; CAHILL, R., COOKSON, R. C. and CRABB, T. A. (1969) *Tetrahedron* **25**, 4681, 4711

[19] GHISALBERTI, E. L. unpublished data

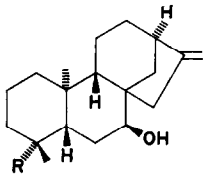
convert C-4$CH_2OH \rightarrow$ C-4CO_2H[9,20] and thus it can be expected to utilize the 7β, 19-diol as for the 7β, 19-oic acid. Although sequences aimed at removing C-3 oxygenation from the triols are readily visualized they are comparatively laborious and clearly could be avoided if analogous hydroxylation could be effected for the *ent*-kaurenyl succinate (**21**). Preliminary experiments with the 3-hydroxy succinate (**7**) had shown that the succinate ester survived the metabolic process and hence might be acting alone as an adequate block to further metabolism of the dihydroxy succinates. *ent*-Kaurenyl succinate (**21**) was prepared by the standard method and added to cultures of *G. fujikuroi*. Saponification of the acidic product and separation of the neutral fraction gave a diol (**22**) and a triol (**24**). While we were determining the structure for the former compound Professor West advised us that his group had shown that a homogenate of the endosperm nucellus of *Echinocystis macrocarpa* converts the *ent*-kaurenoic acid (**4**) to its 7β-hydroxy derivative (**23**)[21] and that the latter compound had been partially synthesized.[22] A sample of the acid (**23**) provided by Dr. Hanson was converted to the 7β,19-diol (**22**) and this was shown to be identical with our metabolic product.



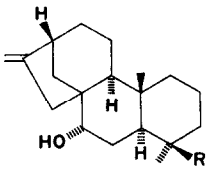
(**20**)



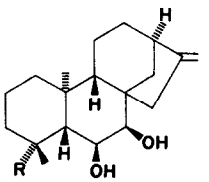
(**21**)



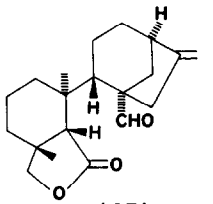
(**22**) R = CH_2OH
(**23b**) R = CO_2H



(**23a**) R = CO_2H



(**24**) R = CH_2OH
(**25**) R = CO_2H

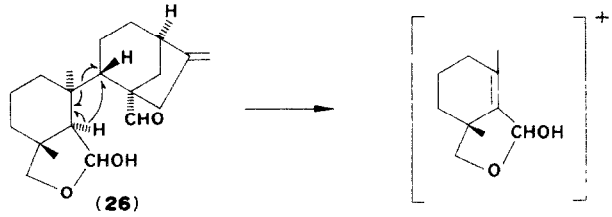


(**27**)

The triol (**24**) had physical data for the introduction of two secondary hydroxyl groups into the *ent*-kaurenol (**2**). In addition the NMR spectrum revealed that the signals for the carbinol methine protons at δ 4·46 (*dd*, C-6 H) and 3·84 (*d*, C-7 H) were mutually coupled (J 2 Hz) and that the former signal was coupled to a sharp doublet (δ 2·22, J 11 Hz) which is assigned to C-5 H. The latter coupling requires the C-6 H to be axial and the small C-6 H:C-7 H coupling then requires the C-7 H to be equatorial. This coupled system of three contiguous protons associated with two secondary hydroxyl groups is accommodated in the kaurene B-ring. Confirmation of the 6,7-diol group followed from reaction of the triol (**24**) with periodate which gave an aldehyde with IR max at 1725 cm^{-1} and a singlet NMR resonance (δ 9·85 due to the formyl group). A broadened singlet which appeared at δ 5·35 was consistent with a hemiacetal proton and on this basis structure (**26**) was assigned to

[20] Cook, I. F., Jefferies, P. R and Knox, J R (1971) *Tetrahedron Letters* 2157
[21] Murphy, P. J and West, C. A (1969) *Arch Biochem Biophys* **133**, 395, Lew, F. T and West, C A (1971) *Phytochemistry* **10**, 2065.
[22] Hanson, J. R., Hawker, J and White, A. F (1972) *J. Chem Soc. Perkin* **I**, 1892

the oxidation product. The B-ring cleaved structure was evident from the MS which showed its base peak at *m/e* 168 which is attributed to an ion resulting from cleavage of the C-9:C-10 bond with loss or transfer of hydrogen (Scheme 3)



SCHEME 4

Added support for the acetal structure was obtained by selective oxidation with silver carbonate[23] which gave the γ-lactone (**27**) retaining the formyl group The lactone (**27**) had spectral properties identical with those obtained by Cross *et al* for a sample derived from fujenal [24]

The production of the 6β,7β,19-triol (**24**) by the metabolic process was particularly interesting since it suggested that gibberellin biosynthesis might involve sequential 7β- and 6β-hydroxylation of a kaurene precursor. 6,7-Dioxygenated kaurenes have been favoured in speculations[10] on the mechanism of the B-ring contraction. Plausible routes from such precursors include benzilic acid-like rearrangement of a 6,7-dione, Favorskii-type reaction of an esterified 6-hydroxy-7-one and pinacolic-type rearrangement of a 6,7-diol or ester Since Cross *et al.*[11] reported negative incorporation studies with 6α,7β- and 6α,7α-dioxygenated systems the 6β,7β-system was an excellent candidate. In order to test this possibility we undertook the preparation of the 17-[^{14}C]-labelled triol (**24**) by the metabolic procedure The [17-^{14}C]-kaurenol (**2**) was obtained by a variant of the reported procedure,[8] esterified with succinic acid and the product fed to *G. fujikuroi* A time study of the metabolism followed by radiochromatography showed that after 2 days the radioactivity was distributed in about equal amounts between gibberellic acid (**6**) and the succinates of the diol (**22**) and triol (**24**). After 7 days the diol succinate had fallen to negligible concentration and radioactivity was distributed in similar proportions between gibberellic acid and the triol succinate The incorporation into gibberellic acid, which was verified by dilution and isolation is apparently due to lability of the succinate function A larger scale metabolism of the labelled succinate (**21**) was carried out for 3 days and processed to give samples of [17-^{14}C]-labelled diol (**22**) and triol (**24**). The labelled diol was then fed to *G fujikuroi* and the gibberellic acid separated, diluted, methylated and crystallized to constant sp act The incorporation into gibberellic acid was ~ 2%. While significant this value is much lower than that (32 3%) which has been reported for the 7β-hydroxy acid (**23**).[22] The methyl gibberellate derived from a similar feeding of the triol (**24**) showed negligible (<0 2%) incorporation suggesting that a 19-oxygenated-6β,7β-dihydroxy kaurene is not an intermediate in gibberellin biosynthesis The same conclusion has been reached by Cross *et al* [25] who prepared the 6β,7β-dihydroxy acid (**25**) by osmylation of a 6-ene. They found that neither the acid (**25**) nor the triol (**24**) were converted to gibberellic acid although the former was an efficient precursor of fujenal These results are consistent with a report[22] requiring that

[23] FETIZON, M and GOLFIER, M (1968) *Compt Rend* **267**, 900

[24] CROSS, B E, GALE, R H B and HANSON, J R (1963) *J Chem Soc* 5052, HANSON, J R and WHITE, A E (1970) *Tetrahedron* **26**, 2711

[25] CROSS, B E, STEWART, J C and STODDART, J L (1970) *Phytochemistry* **9**, 1065

both the 6α and 6β H of the kaurenoid intermediates are retained in the formation of the gibberellin aldehyde (**5**) in *G. fujikuroi*. In contrast with their failure to incorporate into gibberellic acid in the mould these oxygenated kaurenes were found to possess effective gibberellin activity in several plant assays.[25] Other kaurenes which have been found to act as gibberellin precursors have lower activity in these assays[25] and it is possible that the 6β,7β-dihydroxy compounds (**24**) and (**25**) are converted to gibberellins in the higher plants. Although there is good evidence to show that hydroxylation of the gibberellin A-ring occurs after the B-ring contraction step[26] the ready availability of the 3β,6α,19- and 3β,7β,19-triols warranted testing their metabolic fate in the mould. The [17-^{14}C]-labelled compounds were prepared by Wittig reaction of [^{14}C]-triphenylmethylphosphonium iodide with *ent*-17-nor-3α-hydroxykauran-16-one to give [17-^{14}C]-labelled diol (**12**) which was then succinoylated. After incubation with the mould and hydrolysis of the acidic fraction the [17-^{14}C]-triols **16** and **18** were obtained. Neither triol showed significant alteration after separate exposure to *G. fujikuroi*. When the parent 3β,19-diol was metabolized in the mould most of the radioactivity corresponded to the diol but a small proportion (*ca* 5%) showed TLC behaviour for the 3β,6α,19-triol.

The results obtained for the metabolism of *ent*-kaur-16-en-19-yl succinate (**21**) have been reported in a short paper.[27]

EXPERIMENTAL

General details have been described[28] MS were measured by direct insertion using a MAT-CH 7 Mass spectrometer with energies of 10–70 eV and temps from 60–280° NMR spectra were measured with a Varian A60 spectrometer for $CHCl_3$ or $CDCl_3$ solns unless otherwise stated

General fermentation conditions *Gibberella fujikuroi* ACC 917 was grown in shaken culture on a glucose–ammonium nitrate medium (400 ml)[29] in conical flasks (1 l) until inorganic N was exhausted (*ca* 4 days) when the substrate for incorporation studies was added in a small vol. EtOH After the stated time the mycelial mass was filtered, washed with H_2O and the filtrate acidified (pH 2–3) and extracted with EtOAc The acidic fraction was separated from the EtOAc by washing with 8% $NaHCO_3$ (aq.) and recovered with EtOAc Mycelium used in resuspension was filtered after 4 days growth, washed with sterile H_2O and shaken with the substrate (in EtOH) in the growth medium lacking glucose After the stated time the product was fractionated into acidic and neutral portions as described for the other incorporation studies

Metabolism of ent-19-(*3-carboxypropionyloxy*)-*kaur*-16-*en*-3β-*ol* (**7**) The hydroxy succinate (**7**) isolated from *G strophiolata*[12] was added in portions (50 mg) to 12 1-l flasks each containing resuspended *G fujikuroi* mycelium in 400 ml of medium at pH 7 After shaking for 7 days the filtrate was acidified and extracted with EtOAc The extract was evaporated and the product hydrolysed in 10% NaOH (aq) during 1 5 hr at 20° Extraction with EtOAc and crystallization of the product from EtOAc gave ent-*kaur*-16-*ene*-3-β,7α,19-*triol* (**8**, 200 mg) as needles, m p 232–234°, $[\alpha]_D$ − 34 (*c* 0 8, MeOH) (Found C, 74 7, H, 10 3 $C_{20}H_{32}O_3$ requires C, 75 0, H, 10 1%) ν_{max} (Nujol) 3440, 3300 cm^{-1} (OH), 3050, 860 cm^{-1} (vinyl group) NMR (pyridine-d_5, δ) 1 07, 1 56 (*s*, tertiary methyls), 2 57 (*bs*, C-15 H_2), 2 64 (*m*, C-13 H), 3 70 (*m*, C-3 H, C-7 H), AB, δ_A 4 55, δ_B 3·67, *J* 11 Hz (C-19 H_2), 4 91 (*bs*, C-17 H_2) MS *m/e*(%) 320 (M^+, 8), 302 (50), 284 (100), 271 (10), 253 (10), 147 In a preliminary experiment the acidic fraction from the culture filtrate (from 300 mg of **7**) was separated by repeated preparative TLC (PLC) to give the major product (50 mg) which crystallized from petrol–$CHCl_3$ as needles of the succinate of **8** (m p 137–140°) NMR (δ), 1 03, 1 08 (*s*, tertiary methyls), 2 63 (*s*, $COCH_2CH_2CO_2H$), 3 38 ($W_{\frac{1}{2}}$ 16 Hz, C-3 H), *AB*, δ_A 4·16, δ_B 4 38, *J* 11 Hz (C-19 H_2), 4·80 (*bs*, C-17 H_2) Saponification in base gave the triol (**8**)

ent-19-*Benzoyloxykaur*-16-*ene*-3,7-*dione* (**10**) The triol **8** (64 mg) in pyridine (2 ml) was benzoylated with PhCOCl (0 033 ml) for 5 hr at 0° The product was recovered with Et_2O and purified by PLC to give a monobenzoate (**9**, 59 mg) ν_{max} 3600 (OH), 3065 (vinyl H), 1730 cm^{-1} (ester) NMR (δ) 1 08, 1 25 (*s*, tertiary methyls), 2 26 (*s*, C-15 H_2), 2 67 (*m*, C-13 H), 3 43 (*m* C-3 H), 3 68 (*m*, $W_{\frac{1}{2}}$ 6 Hz, C-7 H), *AB*, δ_A 4 58, δ_B 4 42, *J* 12 Hz (C-19 H_2), 4 83 (*bs*, C-17 H_2) MS *m/e*(%) 424 (M^+, 3), 406 (2), 388 (2), 302 (27), 284 (100) The monobenzoate (**9**, 50 mg) in acetone (4 ml) was treated with Jones' reagent (0 10 ml) for 5 min at 0° The product was isolated with Et_2O and crystallized from petrol–$CHCl_3$ as *prisms* (**10**), m p 192–194° $[\alpha]_D$ − 31° (*c* 0 9), λ_{max} 240 nm (EtOH or 8% NaOH, in aq EtOH) ν_{max} 1720 (ester), 1705 cm^{-1} (cyclohexanone) NMR (δ). 1 28, 1 47 (*s*, tertiary

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methyls), *AB*, δ_A 4·89, δ_B 4·23, *J* 11 Hz (C-19 H_2), 4·91 (*s* C-17 H_2), ~8 (phenyl H_5) MS. *m/e*(%), 420 (100), 298 (75), 152 (100); 147 (100), 119 (40) (Found M^+, 420·228 $C_{27}H_{32}O_4$ requires M^+, 420·230) When the diketo benzoate (**10**, 40 mg) was reduced with $NaBH_4$ (9 mg) in diglyme, the product separated by PLC consisted of the monobenzoate (**9**, 5 mg) and an isomer (29 mg) considered to be the 7α-epimer The isomer had the following spectral data. ν_{max} (Nujol). 3520, 3440 (OH), 3070 (vinyl), 1690 (ester) NMR (δ) 1·06, 1·15 (*s*, tertiary methyls), 2·15 (*bs*, C-15 H_2), 2·65 (*m*, C-13 H), 3·41 (*m*, C-3 H, C-7 H), *AB*, δ_A 4·60, δ_B 4·36, *J* 11 Hz (C-19 H_2), 4·82 (*bs*, C-17 H_2), ~8·0 (*m*, phenyl H_5) MS. *m/e*(%), 424 (M^+, 2), 406 (2), 388 (1), 302 (60), 284 (100)

Preparation and metabolism of ent-19-(3 *carboxypropionyloxy*) *kaur*-16-*en*-3α-*ol* (**13**) The diol (**12**, 3 g) obtained by published methods[7] was esterified in pyridine (60 ml) with succinic anhydride (5 g) at 20° during 3 days The product was extracted with Et_2O, washed free of succinic acid with H_2O, and the Et_2O evaporated to give the succinate (**13**) as a resin (3 g) NMR spectrum (δ) 1·03 (*s*, 6H, tertiary methyls), 2·66 (*s*, $COC\underline{H}_2C\underline{H}_2CO_2H$) 3·73 (*m*, $W_{\frac{1}{2}}$ 6Hz, C-3 H), *AB*, δ_A 4·28, δ_B 4·94, *J* 11 Hz (C-19 H_2); 4·77 (*bs*, C-17 H_2) The succinate (900 mg) was metabolized with resuspended *G. fujikuroi* mycelium as for the 3-epimer above After saponification the neutral product was separated by PLC to give three products, the least polar of which was the parent diol (**12**, 97 mg) The intermediate fraction (70 mg) crystallized from acetone as plates of ent-*kaur*-16-*ene*-3α,6β,19-*triol* (**18**), m p 208–210° ν_{max} (Nujol) 3270 (OH), 1660 cm^{-1} (vinyl) NMR (d_5 pyridine, δ) 1·20, 1·91 (*s*, tertiary methyls), 2·67 (*m*, C-13 H), *AB*, δ_A 4·42, δ_B 4·08, *J* 11 Hz (C-19 H_2), 4·50–3·95 (C-3 H, C-6 H), 4·89 (*bs*, C-17 H_2) MS *m/e*(%) 320 (M^+, 1), 302 (M^+ − 18, 66), 284 (100), 271 (40), 253 (40) Found M^+, − H_2O 302·224, $C_{20}H_{30}O_2$ requires 302·224 The most polar fraction crystallized from aq MeOH to give ent-*kaur*-16-*ene*-3β,7α,19-*triol* (**16**) 240 mg as needles m p 185–186°, $[\alpha]_D$ 5° (*c* 0·8, pyridine) ν_{max} (nujol) 3300 (OH), 3065 1660, 870 cm^{-1} (vinyl) NMR (d_5 pyridine, δ) 1·16, 1·59 (*s*, tertiary methyls), 2·62 (*bs*, C-15 H_2), *m*, 3·82 (C-7 H), *AB*, δ_A 4·15, δ_B 3·85, *J* 11 Hz (C-19 H_2); 4·92 (*bs*, C-17 H_2), MS *m/e*(%) 320 (M^+, 4) 302 (81), 284 (66), 271 (50), 253 (100) (Found M^+, 320·235 $C_{20}H_{32}O_3$ requires M^+, 320·235)

ent-19-*Benzoyloxykaur*-16-*ene*-3,7-*dione* (**10**) *from* ent-*kaur*-16-*ene*-3α,7α,19-*triol* (**16**) The triol (**16**), (100 mg) was benzoylated in pyridine (2 ml) with PhCOCl (0·06 ml) during 1 hr at 0° The product was isolated with Et_2O and purified by PLC to give a resinous monobenzoate (**17**, 120 mg) ν_{max} 3620, 3570 (OH), 1725 (ester) NMR (δ) 1·08, 1·19 (*s*, tertiary methyls), 2·29 (*bs*, C-15 H_2), 2·69 (*m*, C-13 H), 3·64 ($W_{\frac{1}{2}}$ 5 Hz, C-7 H), 3·92 ($W_{\frac{1}{2}}$ 4·5 Hz, C-3 H), *AB*, δ_A 4·48, δ_B 4·20, *J* 12 Hz (C-19 H_2), 4·82 (*bs*, C-17 H_2); ~8·0 (*m*, phenyl H_5) MS *m/e*(%) 424 (2), 406 (6), 388 (9), 302 (100), 284 (94), 266 (44) This monobenzoate (**17**, 75 mg) in acetone (5 ml) was oxidized with Jones' reagent (0·25 ml) for 5 min at 0 The product was isolated with Et_2O and crystallized from petrol–$CHCl_3$ as prisms, m p 192–194°, alone or mixed with the diketobenzoate (**10**) described above

ent-3α-*Acetoxy*-6β,19-*epoxykaur*-16-*ene* (**19**) The triol (**18**, 25 mg) in pyridine (2 ml) was treated with tosyl chloride (15 mg) overnight at 0° The product isolated with Et_2O was treated with 2% KOH in MeOH for 2 hr at 20° and extracted with EtOAc PLC gave the hydroxy ether (12 mg) which did not crystallize NMR (δ) 1·06, 1·09 (tertiary methyls) MS *m/e*(%) 302 (M^+, 25), 287 (5), 284 (5), 167 (100), 124 (50) The *acetate* (**19**) crystallized from MeOH as plates, m p 168–169° ν_{max} (Nujol). 3060, 1650 (vinyl), 1725 (acetate) NMR (δ) 1·09, 1·16 (*s*, tertiary methyls), 2·02 (*s*, $COC\underline{H}_3$), *AB*, δ_A 3·78, δ_B 3·48, *J* 8 Hz (C-19 H_2), 4·84 (*bs*, C-17 H_2), 5·20 (*t*, C-3 H) MS *m/e*(%), 344 (M^+, 7%), 284 (26), 209 (100), 150 (95) (Found M^+ 344·235 $C_{22}H_{32}O_3$ requires 344·235 M^+ − $C_{10}H_{15}$, 209·117 $C_{12}H_{17}O_3$ requires 209·118)

Fermentation of ent-3-*oxo*-19-(3-*carboxypropionyloxy*)-*kaur*-16-*ene* (**15**) (*With P. W. Khong*) The ketosuccinate[12] (**15**, 6 × 100 mg) was metabolized with resuspended mycelium of *G. fujikuroi* in 400 ml batches of medium at pH 7 for 3 days The acidic metabolites proved labile and were reduced directly with $LiAlH_4$ in Et_2O Isolation with EtOAc and purification by PLC gave the triol (**7**, 27 mg), m p and m m p 232–233° after crystallization from EtOAc

Preparation and metabolism of ent-19-(3-carboxypropionyloxy) kaur-16-ene (**21**) The *ent*-kaurenoic acid (**4**) was methylated and reduced with $LiAlH_4$ to give *ent*-kaur-16-en-19-ol (**2**) which was succinoylated as described above for the diol (**12**) The *hemi-succinate* (**21**) crystallized from petrol as needles m p 125–127° (Found C, 74·2, H, 9·3 $C_{24}H_{36}O_4$ requires C, 74·2, H, 9·3%) NMR (δ) 0·97, 1·06 (*s*, tertiary methyls), 2·09 (*bs*, C-15 H_2), 2·67 (*s*, 4H-$COCH_2\underline{C}H_2CO_2H$), *AB*, δ_A 4·33, δ_B 3·93, *J* 11·5 Hz (C-19 H_2), 4·80 ($W_{\frac{1}{2}}$ 7 Hz, C-17 H_2) The hemisuccinate was metabolized with resuspended mycelium of *G. fujikuroi* as for (**7**) except that 100 mg substrate was added to each flask and the metabolites separated into acid and neutral fractions after 3 days The former fraction consisted largely of gibberellic acid (**6**) and two succinate metabolites which were enriched by repeated PLC Saponification of the less polar material gave the 7β,19-diol whereas the more polar material hydrolysed to give the 6β,7β,19-triol The metabolism was repeated and the acidic fraction hydrolysed in 10% NaOH (aq) The neutral product formed was separated by PLC to give the *ent*-kaurenol (**2**, 17 mg), the diol (**22**, 36 mg) and triol (**24**, 126 mg) *ent-Kaur*-16-*ene*-7α, 19-*diol* (**22**) crystallized from petrol–acetone as prisms, m p 189–190°, $[\alpha]_D$ − 21 (*c* 1·6) (Found C, 78·9, H, 10·6 $C_{20}H_{32}O_2$ requires C, 78·8, H, 10·6%) ν_{max} (Nujol) 3300 (OH) 3070, 1665, 875 cm^{-1} (vinyl) NMR (δ). 0·98, 1·04 (*s*, tertiary methyls), *AB*, δ_A 3·80, δ_B 3·52, *J* 11 Hz (C-19 H_2), 3·64 (*b*, C-7 H), 4·84 (*b*, C-17 H_2) MS *m/e*(%), 304 (M^+, 5), 286 (42), 255 (100), 123 (91) The sample was identical with material obtained by $LiAlH_4$ reduction of methyl *ent*-7α-hydroxy-kaur-16-en-19-oic acid (**23**) The *triol* (**24**) crystallized from light petrol–$CHCl_3$ as needles, m p 192–193°, $[\alpha]_D$ − 73 (*c* 1·2, MeOH) ν_{max} 3300 (OH), 3055, 1660, 870 cm^{-1} (vinyl) NMR (d_5-pyridine, δ) 1·08, 1·57 (*s*, tertiary methyls), 2·22 (*d*, *J* 11 Hz, C-5 H), 3·84 (*d*, J 2 Hz, C-7 H), 4·46 (*dd*, *J* 11, 2 Hz), *AB*, δ_A 4·39, δ_B 3·75, *J* 11 Hz (C-19 H_2); 4·93 (*m*, C-17 H_2) MS *m/e*(%), 320 (M^+,

2), 302 (100), 284 (40), 271 (77), 253 (55) (Found. M^+, 320.234 $C_{20}H_{32}O_3$ requires M^+, 320.235) The neutral fraction from the metabolism was also treated with 8% NaOH (aq) and the product recovered with EtOAc and chromatographed on Al_2O_3 to give the *ent*-kaurenol (**2**, 56 mg) and the diol (**22**, 45 mg)

Periodate oxidation of ent-*kaur*-16-*ene*-6α,7α,19-triol (**24**) The triol (**24**, 12 mg) in dioxan (4 ml) was oxidized for 12 hr at 20° with 0 5 M $NaIO_4$ (1 2 ml) The neutral product (10 mg) recovered with Et_2O is formulated as the hemiacetal (**26**) ν_{max} 1725 cm^{-1} (CHO) NMR (δ). 0 99, 1 27 (*s*, tertiary methyls), 3 69 (*q*, C-19 H_2), 4 80 (*m*, C-17 H_2), 5 35 (*m*, $W_{\frac{1}{2}}$ 6 Hz, C-6 H), 9 85 (*s*, CHO) MS *m/e*(%), 318 (M^+, 16), 300 (41); 288 (9), 168 (100), 149 (43); 123 (70) The aldehyde (**26**) (5 mg) was refluxed in C_6H_6 with Ag_2CO_3 on celite[23] for 12 hr The product was isolated with Et_2O and purified by TLC to give the lactone aldehyde (**27**, 4 mg) ν_{max} 3060, 875 (vinyl) 2700, 1720 (aldehyde), 1770 cm^{-1} (γ-lactone). NMR (δ): 0 94, 1 13 (*s*, tertiary methyls); *AB*, δ_A 4·05, δ_B 3 79, *J* 9 Hz, (C-19 H_2), 4 88 (*b*, C-17 H_2), 9 80 (*s*, CHO) MS: *m/e*(%), 316 (M^+, 33), 287 (25), 167 (100), 149 (20), 123 (82) The NMR corresponded with that published[24] for authentic material

ent-[17-^{14}C]-19-(3-*carboxypropionyloxy*)*kaur*-16-*ene* (**21**) BuLi (1 8 ml, 2 6 M) was stirred with ^{14}C-MeP^+-Ph_3I^- (1 31 g) in 30 ml Et_2O under N_2 for 30 min and then 0·5 g methyl *ent*-17-nor-16-oxokauran-19-oate in 2 ml Et_2O added The mixture was stirred for 12 hr, H_2O added and the neutral products isolated with Et_2O and chromatographed on Al_2O_3 to give the methyl ester (185 mg) of the *ent*-kaurenoic acid (4) The methyl ester was reduced with $LiAlH_4$ in Et_2O to give the kaurenol (**2**, 142 mg) which was esterified with succinic anhydride as for the cold material to give the *ent*-[17-^{14}C]-kaurenyl succinate (**21**), m p 125–127° (180 mg; 2 06 × 10^5 dpm/mg)

Time study of the metabolism of ent-[17-^{14}*C*]-19-(3-*carboxypropionyloxy*)-*kaur*-16-*ene* (**21**) (*With P W Khong*). Mycelium of *G. fujikuroi* from 60 ml culture medium was resuspended in the same vol of medium lacking glucose and adjusted to pH 7 The succinate (**21**, 10 mg, 2 06 × 10^5 dpm/mg) in EtOH (0 1 ml) was added and the flask shaken for 7 days. Portions (20 ml) were removed in the 2nd, 4th and 7th days and after separation into acidic and neutral fractions, aliquots (2%) were counted Activity in the acidic fractions increased with time from 3 1 × 10^5 through 4 2 × 10^5 to 4 3 × 10^5 dpm with a corresponding reduction in activity of the neutral fractions Portions of the acidic fractions (5%) were submitted to TLC using SiO_2 plates and EtOAc–$CHCl_2$–HOAc (15 5 1) and the plates then scanned for radioactivity The scans for the two days metabolism showed virtually total conversion of the starting material to gibberellic acid (**6**), the succinate of the triol (**24**) and the diol (**22**) After 4 days most of the latter had disappeared and it was not detected in the 7-day acids which consisted of similar proportions of gibberellic acid and the succinate of the triol (**24**) The band for gibberellic acid (**6**) was separated from the balance (93%) of the 7-day acids and the product (2 3 mg) diluted with cold acid (20 mg) and crystallized to constant activity, m p. 228–229°; 1·50 × 10^3 dpm/mg

ent-[17-^{14}*C*]*Kaur*-16-*ene*-7α,19-*diol* (**22**) *and* ent-[17-^{14}*C*]-*kaur*-16-*ene*-6α,7α,19-triol (**24**) The *ent*-[17-^{14}C]-kaurenyl succinate (**21**, 100 mg, 2·06 × 10^5 dpm/mg) was metabolized for 3 days with resuspended mycelium of *G. fujikuroi* as in the previous experiment to give acidic (76 mg, 9 5 × 10^6 dpm) and neutral (20 mg, 1·7 × 10^6dpm) metabolites. The former were hydrolysed in 8% aq NaOH and the neutral product separated by TLC to give the 7,19-diol (**22**, 2·9 × 10^5 dpm) and the triol (**24**, 3 5 × 10^5 dpm) Radioscans of both the diol and triol in different solvent systems showed they were radio-chemically pure

ent-[17-^{14}*C*]*kaur*-16-*ene*-3α,6β,19-*triol* (**18**) *and* ent-[17-^{14}*C*]-*kaur*-16-*ene*-3α,7α,19-*triol* (**16**) *The diol* (**12**, 0 7 g) in dioxan (200 ml) was stirred with OsO_4 (10 mg) and $NaIO_4$ (0 5 M, 65 ml) for 60 hr Isolation with $CHCl_3$ gave the norketo diol which was acetylated with Ac_2O–pyridine. The product (0 1 g) was treated with ^{14}Me P^+ Ph_3I^- as described above. Isolation with ether, basic hydrolysis and PLC gave *ent*-[17-^{14}C]-kaur-16-ene-3α,19-diol m.p. 201–202° (**12**, 49 mg, 3·8 × 10^5 dpm/mg) after crystallization from acetone The diol was esterified with succinic anhydride as for the inactive sample to give the succinate (**13**, 40 mg) as an oil The latter was incubated with resuspended mycelium of *G fujikuroi* in 400 ml of medium at pH 7 1 for 7 days and the metabolites isolated and separated as for the cold material to give the [17-^{14}C]-3,6,19-triol (**18**, 4 6 × 10^5 dpm) and the [17-^{14}C]-3,7,19-triol (**16**, 2·1 × 10^6 dpm).

Fermentation with ^{14}*C-labelled substrates in* G fujikuroi (a) ent-[17-^{14}*C*]*kaur*-16-*ene*-7α,19-*diol* (**22**) The diol (**22**, 7·3 × 10^4 dpm) was incubated for 7 days with a growing 4-day-old culture of the mould The acidic products (14 mg, 2 8 × 10^4 dpm) were methylated (CH_2N_2) diluted with methyl gibberellate (100 2 mg) and crystallized from EtOAc to constant sp act. (13 7 dpm/mg ~2% incorporation) The neutral products (10 mg, 2·02 × 10^4 dpm) gave a radioscan of their TLC plate showing peaks corresponding to *ent*-7α,18-dihydroxy-kaur-enolide and *ent*-kaur-16-ene-6α,7α,19-triol (**24**).

(b) ent[17^{14}*C*]-*kaur*-16-*ene*-6α,7α,19-triol (**24**) The [17-^{14}C]-triol (**24**, 2·1 × 10^5 dpm) was incubated with the mould as in (a) to give inactive methyl gibberellate A radioscan of the TLC plate of the neutral fraction (1 0 × 10^5 dpm) showed largely unmetabolized triol

(c) ent-[17-^{14}C]*kaur*-16-*ene*-3α,19-*diol* (**12**). The diol (**12**, 2·8 × 10^6 dpm) was incubated and worked up as in (a) No activity was observed in the methyl gibberellate but the neutral fraction showed a peak corresponding to the 3β,6α,19-triol (**18**) together with the parent diol When the 3β,6α,19-triol or the 3β,7β,19-triol were treated as in (a) no conversion was observed

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