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POSITION OF THE METABOLIC BLOCK FOR GIBBERELLIN BIOSYNTHESIS IN MUTANT B1-41a OF *GIBBERELLA FUJIKUROI**

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Abstract—Mutant B1-41a, obtained by UV-irradiation of *Gibberella fujikuroi* strain GF-1a, does not metabolise mevalonic acid lactone (MVL), *ent*-kaur-16-ene, *ent*-kaurenol, and *ent*-kaurenal to gibberellins. *ent*-Kaur-16-ene-19-oic acid is completely metabolised to give the same gibberellins in similar concentration as unsupplemented cultures of the parent strain. It is concluded that this mutant is blocked for gibberellin synthesis at the step from *ent*-kaurenal to *ent*-kaurenoic acid. Comparison of the incorporation of MVL into GA_3 by the mutant and the parent strains indicate that the metabolic block is 97·5% effective. A method of preparing *ent*-kaur-16-ene, labelled at C-15 and C-17 by [^{2}H] and [^{3}H] is described.

STUDIES with single-gene mutants of micro-organisms have provided detailed information on primary biosynthetic pathways. A classical example is the elucidation of the shikimic pathway using auxotrophic mutants of *Escherichia coli*.[1,2] A similar approach to the study of secondary metabolic pathways is bedevilled by the technical difficulty of screening for mutants, blocked for the synthesis of metabolites which are not essential for growth. Apart from special cases, for example with pigments such as the tetracyclines,[3] the search for mutants of secondary metabolism is time-consuming since it involves detailed biochemical examination of large numbers of isolates from natural sources or from mutagenic treatment of a wild-type parent strain. In the case of gibberellin (GA) biosynthesis Phinney (unpublished results) has made use of the known GA-induced synthesis and release of α-amylase in embryo-less barley seed[4] to provide a rapid and effective method of screening presumptive mutants of *Gibberella fujikuroi*, blocked for GA-synthesis. Using this method, several presumptive mutants have been isolated and are currently

* Part XI in the series "Fungal Products". For Part X see (1974) *Phytochemistry* **13**, to be published.

[1] DAVIES, B. D. (1955) *Adv. Enzymol.* **16**, 247.

[2] SPRINSON, D. B. (1960) *Adv. Carbohyd. Chem.* **15**, 235.

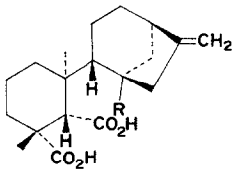
[3] MCCORMICK, J. R. D. (1967) In *Antibiotics* (GOTTLIEB, D. and SHAW, P. D., eds.), Vol. 11, p. 113, Springer, New York.

[4] JONES, R. L. and VARNER, J. E. (1967) *Planta* **72**, 155.

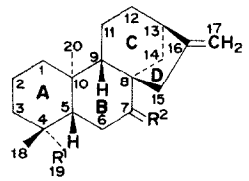
being studied. This paper describes initial studies of a mutant B1-41a, obtained by UV-irradiation of a parent strain GF-1a of *G. fujikuroi.*

A detailed analysis of the diterpenoids from the parent GF-1a by partition chromatography on Sephadex LH20, followed by GC–RC and GC–MS is described elsewhere.[5] From cultures grown on potato-dextrose liquid (PDL) medium, 14 fungal GAs were identified in addition to most of the other known diterpenoid metabolites of *G. fujikuroi.* Although GF-1a and B1-41a grow better on PDL, a synthetic medium was used for reproducibility. The glucose NH_4NO_3 medium (ICI 100%-N) of Borrow *et al.*[6] was used, usually modified by halving the amount of NH_4NO_3 (ICI 50%-N). The metabolites, obtained by EtOAc extraction of the culture filtrates or MeOH extraction of the mycelium were derivatized where necessary and identified by GC–MS.

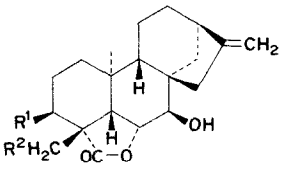
After 36 hr on ICI 50%-N medium, the GF-1a strain showed pigmentation,[7] characteristic of growth where nitrogen had become limiting (the idiophase[8]). At this stage the culture filtrate contained GA_9, GA_{14}, GA_{24}, GA_{25},[9] the di-acid (**1**) and the tri-acid (**2**). After 60 hr these metabolites were still present plus GA_1, GA_3, GA_4, GA_7, GA_{13}, GA_{15} and GA_{16}. After 7 days the only change in the acidic metabolites in the culture filtrate was an increase in the level of GA_3; the neutral metabolites in the culture



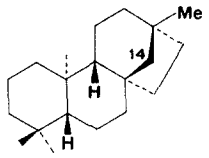
(**1**) R = CHO
(**2**) R = CO_2H



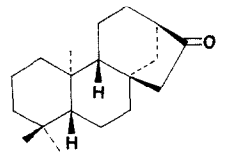
(**3**) R^1 = Me; R^2=H_2
(**4**) R^1 = CH_2OH; R^2=H_2
(**5**) R^1 = CHO; R^2=H_2
(**6**) R^1 = CO_2H; R^2=H_2
(**7**) R^1 = CO_2H; R^2=O



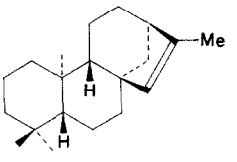
(**8**) R^1 = H; R^2=OH
(**9**) R^1 = R^2=H
(**10**) R^1 = OH; R^2=H



(**11**)



(**12**)



(**13**)

filtrate consisted mainly of 7β,18-dihydroxykaurenolide (**8**) but small amounts of 7β-hydroxy(**9**)- and 3β,7β-dihydroxy(**10**)-kaurenolides were also present. In the mycelial extract after 7 days *ent*-kaurene (**3**) was the major component but traces of *ent*-kaurenol (**4**) and *ent*-kaurenal (**5**) were also present.

[5] MACMILLAN, J. and WELS, C. M. (1974) *Phytochemistry* **13**, to be published.
[6] BORROW, A., BROWN, S., JEFFREYS, E. G., KESSELL, R. H. J., LLOYD, E. C., LLOYD, P. B., ROTHWELL, A., ROTHWELL, B. and SWAIT, J. C. (1964) *Can. J. Microbiol.* **10**, 407.
[7] BORROW, A., JEFFREYS, E. G., KESSELL, R. H. J., LLOYD, E. C., LLOYD, P. B. and NIXON, I. S. (1961) *Can. J. Microbiol.* **7**, 227.
[8] BU'LOCK, J. D. (1967) *Essays in Biosynthesis and Microbial Development*, Wiley, London.
[9] For structures of GAs see MACMILLAN, J. and PRYCE, R. J. (1973) In *Phytochemistry* (MILLER, L. P., ed.), Vol. III, Chap. 11, Van Nostrand-Reinhold, New York.

The mutant B1-41a, grown on ICI 50%-N medium, was indistinguishable from its parent in rate of growth and morphological appearance. After 7 or 11 days no metabolites were detected in the culture filtrate when aliquots of derivatised extract, identical to those used for the parent strain, were examined by GLC. When much larger aliquots were examined traces of GA_3, GA_{13}, the di-acid (**1**) and the tri-acid (**2**) were identified. A comparison of the concentrations of the di-acid (**1**) and GA_3 in the culture filtrates from GF-1a and B1-41a, grown under identical conditions, indicated that *ca* 2% of the di-acid and *ca* 1% of the GA_3 was produced by the mutant. A precise determination of the percentage leakage to GA_3 was obtained as described later. The mycelium from a 5-day old culture of B1-41a on ICI 50%-N medium contained *ent*-kaurene (**3**), *ent*-kaurenol (**4**), and *ent*-kaurenal (**5**). As in the case of the parent GF-1a, *ent*-kaurene (**3**) was the major component but the aldehyde (**5**) while present in amounts higher than in GF-1a did not accumulate in the mutant.

These results indicated that the mutant B1-41a was blocked for GA-synthesis after *ent*-kaurene (**3**) and probably after *ent*-kaurenal (**5**). The position of the block was located by feeding to the mutant [2-$^3H_2$]-mevalonic acid lactone (MVL), *ent*-[15,17-^{3}H]-kaurene, and unlabelled *ent*-kaurenol (**4**), *ent*-kaurenal (**5**), and *ent*-kaurenoic acid (**6**).

When B1-41a was grown on ICI 50%-N medium containing [2-$^3H_2$]-MVL for 6 days, the extract from the culture filtrate contained less than 1·7% of the radioactivity of the 3R-MVL. The EtOAc-soluble portion of the mycelial extract which contained 33% of the radioactivity of the 3R-MVL was examined by TLC on SiO_2 developed first with C_6H_6–PE and then with EtOAc–C_6H_6 in the same direction. Scintillation counting of the material recovered from each 0·5 R_f zone showed that 15% of the activity of this fraction co-chromatographed with *ent*-kaurene (**3**) and *ent*-kaurenal (**5**), 2% with *ent*-kaurenol (**6**), and 35% remained at the origin. The very low incorporation of MVL into GA_3 was also demonstrated by the quantitative determination of the percentage leak as described later. When mutant B1-41a was grown for 5 days on ICI 100%-N medium containing unlabelled MVA, pigmentation did not occur and no *ent*-kaurene (**3**) was detected in the mycelial extract or in the culture filtrate. Thus at least one enzyme for the conversion of MVL to *ent*-kaurene (**3**) is absent or inactive during growth in non-nitrogen limiting media (trophophase[8]).

The mycelial and culture filtrate extracts from B1-41a, grown for 6 days on ICI 50%-N medium containing *ent*-[15,17-^{3}H]-kaurene were examined by TLC as described in the preceding paragraph. In the mycelial extract 58% of the total radioactivity fed co-chromatographed with *ent*-kaurene and 1·2% with *ent*-kaurenal (**5**). In the extract from the culture filtrate, 2% of the radioactivity co-chromatographed with *ent*-kaurene (**3**) and 4% remained at the origin. Thus only 4% of the activity of the substrate was metabolised to more polar compounds. It is also clear from these results that *ent*-kaurene (**3**) occurs predominantly in the mycelium. Thus previously reported incorporations of various precursors into *ent*-kaurene, calculated from *ent*-kaurene isolated from the culture filtrates only, are probably gross underestimates.

At this point, resuspension cultures of B1-41a were used to ensure reproducible mycelium : substrate ratios and to reduce the levels of interfering endogenous metabolites. In this technique which will be discussed in detail in a subsequent paper, the mutant was grown on ICI 50%-N medium until after pigmentation had occurred. The filtered mycelium was washed with fresh medium containing no NH_4NO_3 and adjusted to pH 3·5, then resuspended in this medium containing *ent*-kaurenol (**4**), *ent*-kaurenal (**5**), or *ent*-

kaurenoic acid (**6**). After 20 hr the extracts from the culture filtrates and mycelium were analysed by GLC. From *ent*-kaurenol (**4**) and *ent*-kaurenal (**5**), no metabolites were detected in the culture filtrates and the GLC trace of the derivatised mycelial extract from each feed was identical to that of controls except for the intense peaks of the unmetabolised *ent*-kaurenol (**4**) and *ent*-kaurenal (**5**). In contrast *ent*-kaurenoic acid (**6**) was rapidly and completely metabolised by resuspended cultures of B1-41a. After 20 hr no *ent*-kaurenoic acid was detected in the mycelium or culture filtrate, and the latter was shown by GLC and GC–MS to contain the same metabolites in approximately the same amounts as the culture filtrates of an unsupplemented 7-day culture of the parent strain GF-1a.

These feeding experiments show that the genetic defect in mutant B1-41a interferes with the conversion of *ent*-kaurenal (**5**) into *ent*-kaurenoic acid (**6**). To determine the amount of leakage in the mutant, a comparison was made of the incorporation of MVL into GA_3 by the mutant and the parent strain. Mycelium of each strain in the idiophase was resuspended in ICI 0%-N medium containing [2-^{14}C]-MVL. After 5 days, cold GA_3 was added and the recovered [^{14}C]-GA_3 was purified by TLC and crystallised to constant activity. The incorporation of MVL into GA_3 was 21% for the parent GF-1a and 0·54% for the mutant B1-41a showing that the leakage at the block was $2{\cdot}55 \pm 0{\cdot}1\%$. To correct for the cold GA_3 produced by GF-1a from the unlabelled precursors, the experiment was repeated under the same conditions without added MVL; the unlabelled GA_3 produced was determined by isotope dilution of [^{14}C]-GA_3 added to the culture after 5 days. The corrected percentage leak at the block was $2{\cdot}47 \pm 0{\cdot}1$.

The *ent*-kaurenoid substrates fed to mutant B1-41a were obtained as follows. *ent*-7-Oxokaurenoic acid (**7**) was prepared from 7β-hydroxy kaurenolide (**9**) by the methods of Cross *et al.*[10] and Hanson and Hawker.[11] Wolff–Kishner reduction of the keto-acid (**7**) to *ent*-kaurenoic acid (**6**) required a sealed tube to maintain the temperature recommended by Galt and Hanson.[12] The use of ethylene glycol in place of diglyme allowed a reaction temperature of 215°. However, when the reduction was performed in an open tube, *ent*-kaurenoic acid (**6**) was obtained in low yield and a second product was obtained in 40% yield. The latter compound was identified as a 16,17-dihydro derivative of *ent*-kaurenoic acid by MS and NMR of the Me ester. When the reaction was conducted under N_2, *ent*-kaurenoic acid (**6**) was the sole product. These results suggest that the olefinic double bond had been reduced by di-imide formed by aerial oxidation of hydrazine. $LiAlH_4$ reduction of methyl *ent*-kaurenoate gave *ent*-kaurenol (**4**).[13] Oxidation of the latter with the CrO_3–C_5H_5N reagent in CH_2Cl_2 by the method of Ratcliffe and Rodehorst[14] gave *ent*-kaurenal (**5**) in good yield. The aldehydic proton of *ent*-kaurenal appeared as a doublet (J 1·5 Hz) in the NMR spectrum. In similar compounds this splitting has been attributed[15] to long-range coupling with the 3α- or 6α-protons. However, irradiation at the 4-Me signal (τ 8·99) caused collapse of the aldehydic proton doublet suggesting W-coupling to one of the 4-methyl protons.

ent-Kaur-16-ene (**3**) was labelled at the 15- and 17-positions via the 16-carbonium

[10] CROSS, B. E., GALT, R. H. B. and NORTON, K. (1968) *Tetrahedron* **24**, 231.
[11] HANSON, J. R. and HAWKER, J. (1972) *Tetrahedron* **28**, 2521.
[12] GALT, R. H. B. and HANSON, J. R. (1966) *Tetrahedron* **22**, 3187.
[13] GALT, R. H. B. (1965) *J. Chem. Soc.* 3143.
[14] RATCLIFFE, R. and RODEHORST, R. (1970) *J. Org. Chem.* **35**, 4000.
[15] HENDRICKS, C. A. and JEFFERIES, P. R. (1964) *Australian J. Chem.* **17**, 915.

ion by treatment with [^{2}H]-CF_3CO_2H or [^{3}H]-CF_3CO_2H in C_6H_6. The resulting equilibrium mixture of 15,17-labelled *ent*-kaur-16-ene (**3**) and *ent*-kaur-15-ene (**11**) was separated by TLC. The rate of exchange, followed for [^{2}H] by GC–MS, was slower in DMSO and THF but was faster at increased temperature and acid concentration. Complete exchange was not achieved since a compromise between olefinic and addition products had to be accepted. A typical distribution of deuterium in *ent*-kaur-16-ene was 3%-[$^2H_0$], 8%-[$^2H_1$], 26%-[$^2H_2$], 44%-[$^2H_3$] and 16%-[$^2H_4$]. The [^{2}H]-atoms were located in ring D from the mass spectroscopic fragmentation in which the *m/e* 229 ion from *ent*-kaur-16-ene arises by loss of ring D plus three [^{1}H]-atoms.[16] In the [^{2}H]-compound the *m/e* 230, 231, 232 and 233 were not significantly more intense than those from the unlabelled *ent*-kaur-16-ene. The equal distribution of [^{2}H]-label between the 15- and 17-positions was shown by oxidation to the *nor*-ketone (**12**) which contained 54% of the [^{2}H]. As in the case of the MS of *ent*-kaur-16-ene the unlabelled *nor*-ketone (**12**) fragments with loss of ring D (MeCO, 43 a.m.u.) to give an ion *m/e* 231.[16] In the MS of the [^{2}H]-*nor*-ketone the peaks at *m/e* 232 and 233 are only slightly more intense than those from the unlabelled *nor*-ketone (**12**). These results show that 54% of the [^{2}H]-label in the *ent*-kaur-16-ene is located at the 15-position and 46% at the 17-position. The slightly greater proportion of label in the *nor*-ketone may be due to the formation of *ent*-14-[^{2}H]-beyer-15-ene (**13**) from the treatment of *ent*-kaurene with acid.[17] Tritiation of *ent*-kaur-16-ene (**3**) gave *ent*-[15,17-^{3}H]-kaur-16-ene and the Δ-15 isomer in yields of 15% and 35% respectively. The low yield can be increased by re-cycling and conversion of the 16-alcohols to the olefins.[18]

EXPERIMENTAL

Cultures. Mutant B1-41a and parent strain GF-1a of *Gibberella fujikuroi* were maintained on potato-dextrose-agar (PDA) slants and stored at −5°.

GLC conditions. Me(CH_2N_2) and MeTMS (Sweeley's reagent)[19] derivatives were analysed as follows: (A) Silanised glass column (152·5 × 0·64 cm), 2% QF1 or OV210 on demineralised and silanised Gas Chrom A (80–100 mesh), N_2-flow, 75 ml min^{-1}; (B) 2% SE33 or OV1 as in A; (C) Silanised glass column (152·5 × 0·32 cm), 2% QF1 on demineralised Gas Chrom A (80–100 mesh), N_2-flow, 30 ml min^{-1}; (D) 2% SE33 as in C.

GC–MS. As in Part VII[20] except for GLC conditions.

Culture conditions. Sub-master cultures were grown on PDA slants for 3 days at 25° in light and used to inoculate ICI 50%-N medium (50 ml),[21] in conical flasks (500 ml). The cultures were grown for 4–5 days at 25° under fluorescent light on an orbital shaker at 210 rpm. A mycelium suspension (1 ml) was then taken to inoculate fresh medium (50 ml) in a conical flask (500 ml) which was grown as before for 24 hr. Sub-culturing was repeated and for non-replacement cultures the culture was grown as before for 5–11 days; in re-suspension cultures the mycelium was collected by filtration after 3 days and re-suspended as described later.

Non-replacement cultures. (a) *Metabolism of strain GF-1a.* The culture filtrate from a 7-day culture (50 ml), separated from mycelium by centrifugation at 4000 rpm for 15 min, was adjusted to pH 2·5 with 2N HCl. The EtOAc extract (2 × 100 ml) was partitioned with satd $NaHCO_3$ soln (2 × 100 ml) and the neutral and acidic fractions were recovered in EtOAc in the usual way. The methylated acidic fraction was analysed by GC–MS using the GLC column C, programmed from 200° at 2° min^{-1}. The following metabolites in order of elution were identified: GA_{25}, GA_{24}, GA_9, the tri-acid (2), GA_{13}, the di-acid (1), GA_4, GA_7, fujenal (anhydride of 1), GA_{15}, GA_{16}, GA_1 and GA_3. GC–MS of the MeTMS-derivative of the acidic fraction using

[16] Evans, R. and Hanson, J. R. (1972) *J.C.S. Perkin I* 2382.
[17] Appleton, R. A., McAlees, A. J., McCormick, A., McCrindle, R. and Murray, R. D. H. (1966) *J. Chem. Soc.* **C**, 2937.
[18] Cross, B. E., Galt, R. H. B., Hanson, J. R., Curtis, P. J., Grove, J. F. and Morrison, A. (1966) *J. Chem. Soc.* (C) 2937.
[19] Sweeley, C. C., Bentley, R., Makita, M. and Wells, M. M. (1963) *J. Am. Chem. Soc.* **85**, 2497.
[20] Bearder, J. R., MacMillan, J. and Phinney, B. O. (1973) *Phytochemistry* **12**, 2655.
[21] Geissman, T. A., Verbiscar, A. J., Phinney, B. O. and Cragg, G. (1966) *Phytochemistry* **5**, 933.

GLC column C programmed from 180° at 2° min^{-1} confirmed the presence of GA_{13}, the di-acid (1), the tri-acid (2), GA_4, GA_1, GA_3 and GA_{16}. GC–MS of the TMS-derivatised neutral fraction under the same GLC conditions showed the presence of 7β-hydroxykaurenolide, 3β,7β- and 7β,18-dihydroxykaurenolides. The mycelium was shaken for 24 hr with MeOH (25 ml) then centrifuged. The supernatant was evaporated to dryness under vacuum. The residue was partitioned between aqueous acid (pH 2·5) and EtOAc. The latter was recovered and methylated: GC–MS analysis with column D at 200° showed the presence of *ent*-kaurene (**3**) and traces of *ent*-kaurenol (**4**) and *ent*-kaurenal (**5**). In a time course study GF-1a was cultured on 25 ml of ICI 10%-N medium. The culture filtrates from 36-hr, 60-hr and 7-day culture were extracted and analysed by GC–MS as described above. After 36 hr the culture filtrates contained GA_{25}, GA_{24}, GA_9, the tri-acid (2), GA_{14} and the di-acid (1). After 60 hr the culture filtrate contained the same metabolites as the 7-day cultures, apart from the lower concentration of GA_3.

(b) *Metabolism of mutant B1-41a in 5-day and 11-day cultures.* Cultures (50 ml) were worked up as described in (a). GLC (column A, 200°) of an aliquot (1/2500) of the Me extract from the culture filtrate gave no significant FID peaks although the same aliquot from a control culture of the parent strain GF-1a showed intense peaks of the metabolites identified by GC–MS as described in (a). Aliquots of 1/50 from the B1-41a extract and 1/2500 from the control GF-1a extract gave similar GLC traces. The Me extract from the mycelium was shown by GLC (column B, 200°) and by GC–MS (column D, 200°) to contain *ent*-kaurene (**3**) and traces of *ent*-kaurenol (**4**) and *ent*-kaurenal (**5**).

(c) [2-$^3H_2$]-*MVL feed to B1-41a.* (3-*RS*)-[2-$^3H_2$]-MVL ($1{\cdot}79 \times 10^7$ dpm of *R*-isomer) was added to the medium (50 ml) immediately after autoclaving. After 6 days the culture was worked up as in (a) to give a total culture filtrate extract ($3{\cdot}0 \times 10^5$ dpm, 1·6% incorporation) and a mycelial extract ($5{\cdot}86 \times 10^6$ dpm, 33% incorporation). A portion of the latter was developed on an SiO_2 layer with C_6H_6–PE (3 : 2) for 15 cm and the dried layer was then developed with C_6H_6–EtOAc (9 : 1) for 9·5 cm in the same direction. Bands of silica gel were removed at 0·5 cm intervals and shaken with scintillation fluid which was then counted. Four main zones (dpm) were detected at 0·0–1·0 cm ($1{\cdot}4 \times 10^4$), 6·5–7·5 cm ($5{\cdot}5 \times 10^3$, R_f of *ent*-kaurenal), 8·5–9·5 cm ($3{\cdot}0 \times 10^3$, presumed R_f of olearyl oxide), and 10·0–11·0 cm ($5{\cdot}6 \times 10^3$, R_f of *ent*-kaurene); a small peak ($1{\cdot}3 \times 10^3$) occurred at 2·0–2·5 cm (*ent*-kaurenol).

(d) ent-[15,17-^{3}H]-*Kaurene feed to B1-41a.* The substrate ($7{\cdot}66 \times 10^6$ dpm) was fed to a culture (50 ml) as in (c). The EtOAc extract from the culture filtrate contained $4{\cdot}9 \times 10^5$ dpm (6·4% incorporation) and the mycelial extract contained $4{\cdot}8 \times 10^6$ dpm (62% incorporation). A portion of the mycelial extract was examined by TLC as described in (c): 58% of the radioactivity fed co-chromatographed with *ent*-kaurene and 2·3% with *ent*-kaurenal. TLC and radio-counting of an aliquot of the extract from the culture filtrate in the same way showed that 1·7% of the activity fed co-chromatographed with *ent*-kaurene and 41% remained at the origin.

Re-suspended cultures. The mycelium from the 24 hr sub-culture (50 ml) was washed and re-suspended in ICI 0%-N medium containing KH_2PO_4 (13·6 g l^{-1}). The pH was adjusted to 3·5 with 2N H_2SO_4. The unlabelled substrates (0·5–1·0 mg) were dissolved in the minimum vol. of acetone and added to hot sterile H_2O (5 ml) in a conical flask (100 ml). Mycelial suspension (20 ml) was added and incubated for 20 hr at 25° on a reciprocal shaker. The cultures were worked-up as described for non-replacement cultures.

(a) *Feed of* ent-*kaurenol and* ent-*kaurenal to B1-41a.* GLC (column B, 200°) of the methylated extract from the culture filtrate of each feed gave no significant peaks. GC–MS of the methylated mycelial extract on column D at 210° showed the presence of unmetabolised substrate only.

(b) ent-*Kaurenoic acid feed to B1-41a.* The following metabolites in order of elution were identified by GC–MS of the Me extract from the culture filtrate on column C programmed from 215–230° at 2° min^{-1}: GA_{25}, GA_{24}, GA_9, GA_{14}, the tri-acid (**2**), GA_{13}, the di-acid (**1**), GA_4, GA_7, 7β-hydroxykaurenolide, fujenal, GA_{16}, GA_1 and GA_3.

(c) (3-*RS*)-[2-^{14}C]-*MVL feeds to B1-41a and GF-1a.* The [^{14}C]-MVL ($2{\cdot}1 \times 10^6$ dpm of *R*-isomer) was included in re-suspension medium which had pH 4·8. After 5 days' incubation 120·2 and 143·2 mg GA_3 were added to the B1-41a and GF-1a cultures respectively. The EtOAc (3 × 50 ml) extracted material was subjected to TLC on SiO_2 with EtOAc–$CHCl_3$–AcOH (15 : 5 : 1). The GA_3, recovered from R_f 0·40 in wet EtOAc, was crystallised (×4) from Me_2CO–PE to give successive dpm (±2%) of 175, 122, 117 and 117 from B1-41a (0·54% incorporation) and of 4522, 3922, 3765 and 3830 from GF-1a (21% incorporation). Thus the incorporation into GA_3 from MVL in B1-41a was 2·55 ± 0·1% of that in GF-1a.

(d) *Control incubation for (c).* Cultures were grown as (c) except that [2-^{14}C]-MVL was not included in the re-suspension medium. After 5 days incubation of re-suspended mycelium of B1-41a and GF-1a, 49·1 and 50·4 mg of [^{14}C]-GA_3 (301·9 dpm/mg) were respectively added and the culture filtrates were worked up as in (c). From B1-41a the recovered GA_3 had a specific activity of 299·2 ± 3 dpm/mg (no significant dilution). From GF-1a the recovered GA_3 had 269·9 ± 3 dpm/mg; this dilution corresponded to the production of 5·3 ± 0·2 mg of cold GA_3. The corrected amount of cold GA_3 present in the diluted culture of GF-1a in (c) was thus 148·5 ± 0·2 mg and the corrected yield of GA_3 from B1-41a was 2·47 ± 0·1% of that from the parent strain GF-1a.

ent-*Kaur*-16-*en*-19-*oic acid* (**6**). Following the procedure of Galt and Hanson[12] but replacing the diglyme with ethanediol, *ent*-7-oxokaurenoic acid (18 mg, m.p. 198–201°) in ethanediol (2 ml) and hydrazine hydrate

(0·5 ml) were heated at 150° for 3 hr. KOH (0·3 g) was added and the mixture heated at 215–225° for 3 hr. Work-up in the usual way gave two products (GLC of Me esters) separated by TLC on 10% $AgNO_3$–SiO_2 with EtOAc–PE (3 : 7) into *ent*-kaur-16-en-19-oic acid (8 mg) m.p. 165–166° (lit.[11] m.p. 166–167°), identified by NMR of Me ester, and the 16-epimers of *ent*-kauran-19-oic acid isolated as the Me esters (8 mg); *m/e* 318, 303, 280, 275, 259 and 244; τ 9·18 (3H, *s*), 9·01 (1·5H, *d*, *J* 6 Hz), 8·81 (1·5H, *d*, *J* 6 Hz) and 6·38 (3H, *s*). The above reaction under N_2 gave *ent*-kaurenoic acid (22 mg) from *ent*-7-oxokaurenoic acid (43 mg).

ent-*Kaur*-16-*en*-19-*al* (**5**). *ent*-Kaur-16-en-19-ol (10 mg, m.p. 138–140°), prepared[9] by reduction of Me *ent*-kaurenoate with $LiAlH_4$, was dissolved in CH_2Cl_2 (10 ml) and treated with CrO_3–C_5H_5N reagent (0·1 ml) for 15 min at 20° (see Ref. 14). The mixture was washed with 0·2M NaOH, then H_2O. Recovery gave *ent*-kaurenal (7 mg) m.p. 112·5–114° (from EtOH–H_2O) (lit.[22] m.p. 114°) identified from its MS;[20] τ 9·12 (3H, *s*, 20-H_3), 8·99 (3H, *s*, 18-H_3), 7·83 (2H, *br*, 15-H_2), 7·34 (1H, *br*, 13-H), 5·24 and 5·20 (each 1H, *br*, 17-H_2), 0·35 (1H, *d*, *J* 1·5 Hz, 19-H).

Deuteration of ent-*kaur*-16-*ene*. *ent*-Kaur-16-ene (35 mg) was dissolved in 2% [^{2}H]-CF_3CO_2H–C_6H_6 (10 ml). After 13 hr at 20° satd KH_2PO_4 soln was added and the organic phase was diluted with PE, then washed with H_2O (×3). Evaporation under vacuum gave an oil, purified by TLC on SiO_2 with PE. The mixture (22 mg; 1 : 3) of *ent*-kaur-16-ene and *ent*-kaur-15-ene, recovered from R_f 0·75 was dissolved in THF (20 ml) to which OsO_4 (7 mg) and $NaIO_4$ (500 mg) in H_2O (10 ml). After 2 days at 20° with stirring the THF was distilled off and the aqueous residue, to which more H_2O was added, was extracted with EtOAc. The oil (19 mg), recovered from the EtOAc extract, was analysed by GLC and GC–MS (column C at 180°), showing the presence of *ent*-17-norkauran-16-one. From the intensities of the M^+ and M^+-15 clusters by GC–MS, the norketone had *ca* 54% of the [^{2}H]-content of the *ent*-kaurene from which it was derived.

Tritiation of ent-*kaur*-16-*ene*. *ent*-Kaur-16-ene (25 mg) was dissolved in 2% [^{3}H]-CF_3CO_2H–C_6H_6 (10 ml). After 13 hr at 20° the reaction mixture was worked up as in the previous experiment. The product (26 mg) was purified by TLC on SiO_2 in PE and the material recovered from R_f 0·75 was re-chromatographed on a 10% $AgNO_3$–SiO_2 layer in C_6H_6–PE (3:2). Recovery of the material at R_f 0·3 and 0·6 gave [^{3}H]-labelled *ent*-kaur-16-ene (4 mg) and *ent*-kaur-15-ene (9 mg) identified by GLC and GC–MS. The *ent*-[15,17-^{3}H]-kaur-16-ene was chemically pure by TLC and GLC and radio-chemically pure by GC–RC. The sp. act. by scintillation counting was 6×10^3 dpm/mg.

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