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ENT-KAURENE, OCCURRENCE AND METABOLISM IN *HORDEUM DISTICHON*

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Key Word Index—*Hordeum distichon*; Gramineae; barley; *ent*-kaur-16-ene; estimation; metabolism; *ent*-kaur-16-ene-[^{14}C]; *ent*-kauran-17-ol; *ent*-kauran-16,17-diol; *ent*-kauran-16,17-epoxide.

Abstract—Barley grains contain hydrocarbons, including a material indistinguishable from *ent*-kaurene by GLC, and which after appropriate chemical conversions contain material behaving like *ent*-kauran-16,17-diol, *ent*-kaurene norketone and *ent*-17-nor-kaurane on TLC and GLC. The presence of *ent*-kaurene was confirmed by conversion to *ent*-kauran-16-ol and, following formation of acetate-[^{3}H], recrystallization to constant specific activity with unlabelled carrier. In the initial *ca.* 15 hr of germination, preceding the rise in endogenous gibberellins, the level of *ent*-kaurene falls. Exogenous *ent*-kaurene-[^{14}C] was not metabolized by intact barley grains. *ent*-Kauran-16,17-epoxide was formed non-enzymically by boiled extracts. Unboiled homogenates also formed *ent*-kauran-17-ol and *ent*-kauran-16,17-diol. The diol appeared to be formed from the epoxide, but the *ent*-kauran-17-ol was not. No recognized gibberellin precursors were detected. Nevertheless, endogenous *ent*-kaurene may be the stored biosynthetic precursor of gibberellins in germinating barley grains.

INTRODUCTION

ALTHOUGH the level of gibberellins, especially gibberellic acid† (GA_3,I), increases during barley germination exogenous mevalonic acid-[2-^{14}C] is not incorporated into them.[1,2] Possibly gibberellins are formed from a stored biosynthetic precursor. *ent*-Kaurene (II), a known precursor of gibberellins,[3] is found in many plant tissues.[4–8] Although *ent*-kaurene has been reported to occur in barley grains,[9] the criterion used was the occurrence of a hydrocarbon able to induce α-amylase activity in degermed grains.[9] In contrast to other workers[10,11] we never find *ent*-kaurene has this gibberellin-like property.[12,13] Thus the characterization seemed inadequate, so unambiguous identification was attempted as a preliminary to metabolic studies.

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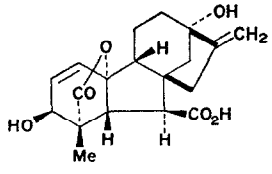
† *Abbreviations*: GA_3—gibberellic acid (I); *ent*-kaurene—*ent*-kaur-16-ene (II); *ent*-kaurene norketone—*ent*-16-oxo-17-norkaurane (IV); *ent*-kaurenol—*ent*-kaur-16-en-19-ol (VII); *ent*-kaurenoic acid—*ent*-kaur-16-en-19-oic acid (VIII); *ent*-kaurenal—*ent*-kaur-16-en-19-al (IX).

[1] GROAT, J. I. and BRIGGS, D. E. (1969) *Phytochemistry* **8**, 1615.
[2] MURPHY, G. and BRIGGS, D. E. (1973) *Phytochemistry* **12**, 1299.
[3] GRAEBE, J. E., DENNIS, D. T., UPPER, C. D. and WEST, C. A. (1965) *J. Biol. Chem.* **240**, 1847.
[4] BRIGGS, L. H., CAMBIE, R. C. and DAVIS, B. R. (1963) *J. Chem. Soc.* 1345.
[5] ALPIN, R. T., CAMBIE, R. C. and RUTLEDGE, P. S. (1963) *Phytochemistry* **2**, 205.
[6] APPLETON, R. A. and CRINDLE, R. (1968) *Phytochemistry* **7**, 135.
[7] PIOZZI, F., PASSANNANTI, S. and PATERNOSTRO, M. P. (1971) *Phytochemistry* **10**, 1164.
[8] DURLEY, R. C., MACMILLAN, J. and PRYCE, R. J. (1971) *Phytochemistry* **10**, 1891.
[9] PETRIDIS, C., VERBEEK, R. and MASSART, L. (1965) *J. Inst. Brewing* **71**, 469.
[10] JONES, K. C. (1968) *Planta* **78**, 366.
[11] CROSS, B. E., STEWART, J. C. and STODDART, J. L. (1970) *Phytochemistry* **9**, 1065.
[12] BRIGGS, D. E. (1966) *Nature* **210**, 419.
[13] MURPHY, G. CLUTTERBUCK, V. and BRIGGS, D. E. unpublished.

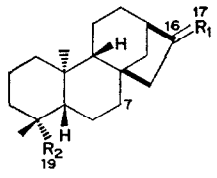
RESULTS

ent-*Kaurene from Barley*

A marker of *ent*-kaurene-[^{14}C] (75 000 dpm, 230 ng), undetectable by GLC, was added to the neutral lipid from decorticated barley (10 kg), and the non-saponifiable fraction was recovered. A hydrocarbon fraction was prepared by chromatography on two successive alumina columns. On GLC in several systems material was present which moved with authentic *ent*-kaurene. About 620 μg was present in the hydrocarbon fraction.



(I) Gibberellic acid, GA_3



(II) R_1 = $=CH_2$; R_2 = $-Me$
(III) R_1 = $-OH, -CH_2OH$; R_2 = $-Me$
(IV) R_1 = $=O$; R_2 = $-Me$
(V) R_1 = $-H, -H$; R_2 = $-Me$
(VI) R_1 = $-OH$, Me; R_2 = $-Me$
(VII) R_1 = $=CH_2$; R_2 = $-CH_2OH$
(VIII) R_1 = $=CH_2$; R_2 = $-CO_2H$
(IX) R_1 = $=CH_2$; R_2 = $-CHO$
(X) R_1 =  R_2 = $-Me$
(XI) R_1 = $-H, -CH_2OH$; R_2 = $-Me$
(XII) R_1 = $-H, -CHO$; R_2 = $-Me$
(XIII) R_1 = $-H, -CO_2H$; R_2 = $-Me$
(XIV) R_1 = $-H, -OH$; R_2 = $-Me$

Most of the hydrocarbon fraction was treated with osmium tetroxide to convert any *ent*-kaurene (II) to *ent*-kauran-16,17-diol (III). The diol was purified by TLC (solvent 1) the zone being located by its radioactivity. GLC with two systems demonstrated a substance with the retention time of authentic *ent*-kauran-16,17-diol.

The material was further treated with periodate to convert presumptive *ent*-kauran-16,17-diol (III) to *ent*-kaurene norketone (IV). The norketone was purified by TLC (solvent 7). Material was present which on GLC in two systems had the same retention time as *ent*-kaurene norketone (IV).

The preparation was reduced (Wolff–Kishner) to convert any *ent*-kaurene norketone (IV) into *ent*-17-norkaurane (V). The hydrocarbon products were purified by TLC (solvent 3). On GLC material with the retention time of authentic *ent*-17-norkaurane (V) was observed in two systems.

The TLC and GLC results all indicated that *ent*-kaurene had been present in the initial extracts. However, although on GLC each treatment produced material with a well-defined peak at the retention time of the appropriate standard many other peaks were obtained. Thus confirmation of the presence of *ent*-kaurene was sought by other means.

Lipid was extracted from ground barley (200 g) and *ent*-kaurene-[^{14}C] was added as a marker. A radioactive hydrocarbon fraction was purified and was treated to convert *ent*-kaurene (II) into *ent*-kauran-16-ol (VI). The alcohol fraction was purified, then acetylated with acetic anhydride-[^{3}H] (1 mCi). The crude acetate esters were separated by TLC, and added to unlabelled *ent*-kauran-16-ol acetate. Constant specific activity was achieved on successive recrystallizations as was equality of specific activity in crystals and materials from their supernatant liquids (e.g. 6th crystals, 6·1 mg, [^{3}H], 17 514 dpm/mg; material from the 6th supernatant, 2·9 mg; [^{3}H], 17 143 dpm/mg). Thus radioactive *ent*-kauran-16-ol-acetate-[^{3}H] was present, and as the activity was much greater than could be accounted for with the [^{14}C]-*ent*-kaurene marker the barley must have contained *ent*-kaurene.

Changes in ent-*Kaurene During Germination*

If the *ent*-kaurene detected in barley is a stored biosynthetic precursor of gibberellins, then on germination the quantity present should decrease. Lipids were extracted from barley samples germinated for various times. Traces of *ent*-kaurene-[^{14}C] were added to aid in isolation and to enable corrections to be made for losses. Hydrocarbon fractions were isolated, treated to convert any *ent*-kaurene (II) to *ent*-kaurene norketone (IV) and were separated by TLC (solvent 2) and assayed by GLC. This procedure was necessary as *ent*-kaurene moved close to another hydrocarbon on direct GLC. Repeated trials showed a sharp decline in *ent*-kaurene in the initial stages of germination, which then levelled out (Fig. 1). In two trials there were slight indications of a transitory increase in the level of *ent*-kaurene at about 25 hr.

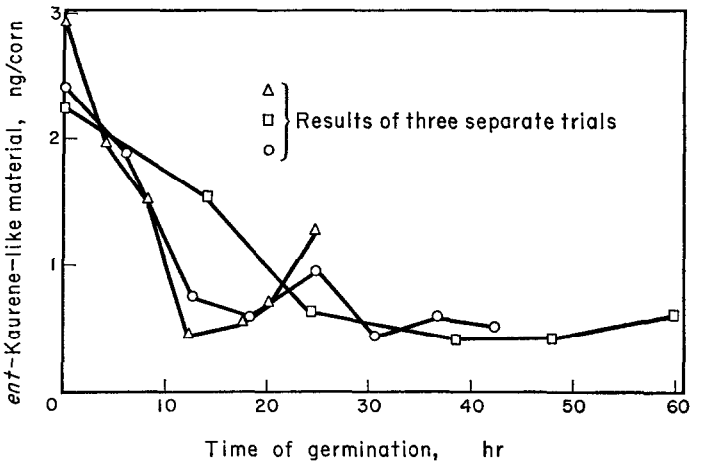


FIG. 1. LEVELS OF *ent*-KAURENE, DETERMINED BY GLC OF *ent*-KAURENE NORKETONE, DURING BARLEY GERMINATION

Losses caused by conversion of the hydrocarbon to the norketone were determined radiochemically.

Metabolism of ent-*Kaurene*-[^{14}C] *by Germinating Barley*

The ability of germinating, decorticated barley grain (100 g dry wt) to metabolize exogenous *ent*-kaurene-[^{14}C] (II) was tested. Grain was grown for 2·5 days, when the maximal level of endogenous gibberellins occurs.[1] The surface was rinsed with isopropanol to remove adhering *ent*-kaurene-[^{14}C], and the lipid fraction was prepared. The isopropanol washings and the lipid extract were each separated into acidic and neutral fractions (Table 1). When the fractions from the isopropanol wash were subjected to TLC (neutral fraction, solvent 4; acidic fraction solvents 5, and then 6) all radioactivity moved with *ent*-kaurene. The radioactivity of the acidic fraction of the barley lipid all moved with *ent*-kaurene on TLC (solvent 5, to remove non-polar material, then solvent 6). The neutral fraction of the barley lipid was saponified, ether-soluble fractions were recovered and separated by TLC (solvent 4). Radioactivity moved with *ent*-kaurene, none was found with the mobility of the biosynthetic intermediates *ent*-kaurenol (VII) or *ent*-kaurenoic acid (VIII).

In attempts to increase the amount of *ent*-kaurene-[^{14}C] penetrating the grain it was found that immersion in a solution of *ent*-kaurene-[^{14}C] in dichloromethane[14] prevented germination, while application of the *ent*-kaurene-[^{14}C] adsorbed onto bovine serum albumin gave

[14] MEYER, H. and MAYER, A. M. (1971) *Science* **171**, 583.

no better penetration. After germination radioactivity was again confined to the hydrocarbon fraction.

TABLE 1. RADIOACTIVITY IN LIPID FRACTIONS FROM BARLEY (100 g) GERMINATED IN THE PRESENCE OF *ent*-KAUR-16-ENE-[^{14}C] (3×10^5 dpm)

Fraction	Weight recovered (mg)	Radioactivity recovered (dpm)
Isopropanol wash		
(a) Neutral lipid	18 5	233 600
(b) Acidic fraction	2·6	1600
Lipid extract		
(a) Neutral lipid	2580	61 500
(b) Acidic fraction	27·3	4500
(c) Neutral saponified	125	57 340
(d) 5% Na_2CO_3 insoluble	22·0	1690

The Metabolism of ent-*Kaurene-*[^{14}C] *by Embryo Extracts*

The microsomal fractions of extracts prepared from several plants actively synthesizing gibberellins contained mixed function oxidases which transform *ent*-kaurene to more oxidised products on the gibberellin biosynthetic path.[15–17] With this in mind embryos were separated from decorticated, sterilised barley after 37 hr germination. Cell-free preparations were made and divided into a 'microsomal' fraction and a microsome-free supernatant fraction. *ent*-Kaurene-[^{14}C] was incubated with these preparations, with added NADPH. Lipid extracts of the incubation mixtures were analysed, when three radioactive materials, *A*, *B* and *C*, separated from *ent*-kaurene on TLC (solvent 4). Compound *B* had the same mobility as *ent*-kaurenol (VII) while compound *C* moved near to *ent*-kaurenal (XI). Compound *A* moved with *ent*-kauran-16,17-diol. Compound *C* was formed about equally by the microsomal and supernatant preparations, while smaller amounts of *A* and *B* were formed by the microsomal preparations.

ent-Kaurene (II, 290 000 dpm) was incubated with a microsomal preparation and the products were purified by TLC (solvent 4). Typical radiochemical yields, from 1 hr incubations at 25°, were, compound *A*, 4·1%; compound *B*, 3·3%; compound *C*, 14·6%. Compound *C* was unchanged by an oxidizing treatment with alkaline silver nitrate (TLC, solvent 7), although *ent*-kaurenal (IX) was converted to *ent*-kaurenoic acid (VIII). Treatment with dilute acid in methanol, in an attempt to hydrate the 16,17-double bond, caused no change in mobility (TLC, solvent 4) suggesting that this had already reacted. Few oxidised derivatives of *ent*-kaurene have the high mobility of compound *C* (TLC, solvent 2). *ent*-Kauran-16,17-epoxide (X) does, and it co-chromatographed with compound *C* (TLC, solvents 2 and 4). Reduction of compound *C* or authentic *ent*-kauran-16,17-epoxide (X) with sodium dihydro-*bis*-(2 methoxy-2-ethoxy) aluminate gave a product moving with *ent*-kauran-16-ol (VI; TLC, solvent 4). Compound *C* was treated with boron trifluoride in ether, and the product was reduced with sodium borohydride. Products were separated by TLC (solvent 4). Compound *C* reacted as authentic *ent*-kauran-16,17-epoxide[18] (X) and

[15] DENNIS, D. T. and WEST, C. A. (1967) *J. Biol. Chem.* **242,** 3293.
[16] COOLBAUGH, R. C. and MOORE, T C (1971) *Phytochemistry* **10,** 2401.
[17] MURPHY, P. J. and WEST, C. A. (1969) *Arch. Biochem. Biophys.* **133,** 395.
[18] HENBEST, H. B. and WRIGLEY, T I (1957) *J Chem. Soc.* 4596.

gave rise to an aldehyde on ring opening, which could be reduced by sodium borohydride to an alcohol, presumably *ent*-kauran-17-ol[19] (XI; TLC, solvent 4). When compound *C* was further incubated with an embryo extract a product moving with *ent*-kauran-16,17-diol (III) was formed (TLC, solvent 4). The identity of the diol was confirmed by conversion of the radioactive substance to a material moving with *ent*-kaurene norketone (IV) by periodate treatment (TLC, solvent 4).

Compound *B*, together with added *ent*-kaurenol (VII), formed a radioactive 4-(4'-nitrophenylazo)benzoyl ester, confirming that it was an alcohol. On recrystallization from benzene, radioactivity declined to zero, showing that compound *B* was not *ent*-kaurenol (VII), the intermediate in gibberellin biosynthesis, although the derivatives co-chromatographed (TLC, solvent 8).

When compound *B* was treated with dilute acid, in an attempt to hydrate the 16,17-double bond, if present, the mobility was unchanged (TLC, solvent 9). Thus the double bond of *ent*-kaurene (II) was already changed, so compound *B* must have been *ent*-kauran-16-ol (VI) or *ent*-kauran-17-ol (XI). It was *ent*-kauran-17-ol (XI) since oxidation with Jones' reagent gave some material chromatographing with *ent*-kauran-17-al, (XII) (TLC, solvent 4) a type of product that cannot be obtained from *ent*-kauran-16-ol (VI). Further confirmation was provided by oxidation of XII with alkaline silver nitrate which gave an acid thought to be *ent*-kauran-17-oic acid (XIII), which moved as expected on TLC (solvent 4), both before and after methylation.

Compound *A* was suspected to be *ent*-kauran-16,17-diol (III) because of its low mobility (TLC, solvent 4) and because of the co-occurrence of *ent*-kauran-16,17-epoxide (X). This was confirmed since treatment with periodate gave material chromatographing with *ent*-kaurene norketone (IV; TLC, solvent 4). As with authentic *ent*-kaurene norketone reduction of the radioactive material gave a product with the mobility of *ent*-16-hydroxy-17-norkaurane (XIV; TLC, solvent 4). Acetylation of compound *A* gave a material chromatographing with the diacetate of *ent*-kauran-16,17-diol (TLC, solvent 4).

It later appeared that no soluble cofactors were required for the conversion of *ent*-kaurene to compounds *A*, *B* or *C* by microsomal or microsomal supernatant preparations. Boiled preparations did not produce *ent*-kauran-16,17-diol (III) or *ent*-kauran-17-ol (XI) but the formation of *ent*-kauran-16,17-epoxide (X) was unaffected indicating that formation is non-enzymic. Lipid extracted from a cell free barley homogenate was incubated with *ent*-kaurene-[^{14}C] in methanol, while illuminated by a bulb, (100 W, 15·2 cm distance), and gassed with O_2. *ent*-Kauran-16,17-epoxide (X) was formed, clearly indicating that non-enzymic synthesis could occur. It is known that fungal pigments are involved in the photochemical formation of peroxide from unsaturated compounds.[20] Thus homogenates probably convert *ent*-kaurene (II) to *ent*-kauran-16,17-epoxide (X) non-enzymically, which is changed into III by enzyme-catalysed reactions. *ent*-Kauran-17-ol (XI) is not produced from X. Presumably the formation of XI from *ent*-kaurene (II) is due to a direct hydration of the double bond.

DISCUSSION

ent-Kaurene is present in barley, and the amount disappearing in the initial decline, (*ca.* 2 ng/grain) is more than sufficient to account for the observed rise in levels of gibberellin-like materials (*ca.* 1 ng GA_3 equivalent/grain).[1] Thus *ent*-kaurene may be the major store

[19] HANSON, J. R. (1967) *Tetrahedron* **23**, 801.
[20] ADAM, H. K., CAMPBELL, J. M. and CORKINDALE, N. J. (1969) *Nature* **216**, 397.

of biosynthetic gibberellin precursor. It is supposed that it is stored in the scutellum and that enzymes convert it into gibberellins which are generally released to trigger enzyme production in the aleurone layer.[21,22] The increase in *ent*-kaurene observed in two trials suggests that after 24 hr some *ent*-kaurene synthesis is occurring, but as exogenous mevalonic acid-[2-^{14}C] is not incorporated into GA_3,[2] the nature of this increase requires further investigation.

The conversion by embryo extracts of *ent*-kaurene into materials with chromatographic properties similar to the gibberellin biosynthetic intermediates is a hitherto neglected complication. The formation of *ent*-kauran-17-ol in homogenates, rather than the 16-homologue was at first surprising, but various 17-oxygenated derivatives of *ent*-kaurene are reported to occur.[23,24] The failure to demonstrate incorporation of *ent*-kaurene-[^{14}C] into gibberellin intermediates in extracts or whole grains is not unexpected with such a water-insoluble compound. Others have failed to detect incorporation of *ent*-kaurene-[^{14}C] into gibberellin intermediates unless the precursor is generated *in situ* from mevalonic acid-[2-^{14}C].[16] Barley extracts are unable to form *ent*-kaurene.[2] It seems that exogenous *ent*-kaurene does not reach metabolic sites in intact barley grains although it will penetrate and be metabolized by *Pharbitis nil* seedlings.[25,26] The later gibberellin precursors, *ent*-kaurenol (VII) and *ent*-kaurenoic acid (VIII) do penetrate barley grains and are converted into other substances.[27]

EXPERIMENTAL

Germination of barley. The selection and decortication of barley grains, cv. Proctor, and the aseptic precautions employed have been described.[1] The germination conditions used approximated to the commercial malting process. Decorticated sterile grain (100 g dry wt) was soaked in sterile H_2O (24 hr, 14·4°) drained (1 hr) then soaked (24 hr). After a second draining period the flask was placed on its side and rotated to spread out the grain. During germination, at 14·4° in the dark, the grain was occasionally stirred to prevent the roots matting When *ent*-kaurene-[^{14}C] was added to the intact grain the solution was sterilized by passage through a Millipore filter, added to the barley after the second draining period and spread evenly by agitation The radioactivity of the solution was determined after Millipore filtration.

Preparation of cell-free extracts. Germinated grain (30 g) with a sucrose solution (70 ml 710 g sucrose/l.) was homogenized for 30 sec at half max. speed in an MSE top-drive homogenizer.[28] The suspension was centrifuged (1000 *g* 5 min). Floating, separated embryos were decanted. The sucrose solution was removed by filtration Embryos (2–5 g) were prepared from 200 g of dry grain Embryos and insoluble polyvinyl pyrrolidone (0·5 g/g embryos) were added to buffer (40 ml, 0 1 M Tris–HCl, pH 7·1, with $MgCl_2$, 5 mM, and 2-mercaptoethanol, 10 mM) and were lightly ground with a pestle and mortar, then at 800 rpm in a Potter homogenizer with 0 15 mm radial clearance. The homogenate was filtered through two layers of nylon mesh (15 denier), then centrifuged (10 000 *g* 15 min). The supernatant was decanted and re-centrifuged (100 000 *g* 1 hr) to give a microsomal pellet. The supernatant material precipitating between 20 and 80% satn with $(NH_4)_2SO_4$ was retained, dissolved in buffer (15 ml) and dialysed against the original buffer. Typical protein contents of extracts *ca* 6 mg/ml The microsomal pellet was resuspended in buffer.

Incubation of cell-free embryo extracts with ent-*kaurene*-[^{14}C] Incubations typically contained *ent*-kaurene-[^{14}C], 58 000 dpm, NADPH (1 mM), and protein (5·2 mg) in the preparation buffer (1·0 ml) and lasted for 1 hr at 25°.

Lipid extraction from cell-free preparations. Me_2CO (equal vol) was added to stop incubations. Et_2O (1·5 × original vol.) was added, liquids were mixed, then separated by centrifugation. Organic phase was collected. Et_2O extraction was repeated (×2) Pooled Et_2O extracts were washed with H_2O (×3) then dried with Na_2SO_4 (1 g/4 ml).

[21] RADLEY, M. (1969) *Planta* **86,** 218.
[22] BRIGGS, D. E (1972) *Planta* **108,** 351
[23] JEFFERIES, P. R. and PAYNE, T. E. (1965) *Australian J. Chem.* **18,** 441.
[24] ESHIET, I. T. U. AKISANYA, A. and TAYLOR, D. A. H. (1971) *Phytochemistry* **10,** 3294.
[25] TAKEBA G. and TAKIMOTO, A. (1971) *Plant Cell Physiol.* **12,** 81.
[26] BARENDSE, G. W. M. and KOK, N. J. J. (1971) *Plant Physiol.* **48,** 476
[27] MURPHY, G. and BRIGGS, D. E. in preparation.
[28] JOHNSTON, F. C. and STERN, H. (1957) *Nature* **179,** 160.

Lipid extraction from intact grain grown with ent-*kaurene*-[^{14}C]. Grain (100 g dry wt) was rinsed with iso-PrOH (3 × 300 ml), then homogenized with iso-PrOH (400 ml, 2 min, top speed Waring blender). Slurry was left for 30 min, with occasional mixing, then filtered. Debris were re-extracted with iso-PrOH (400 ml), then iso-PrOH–$CHCl_3$ (1:1, 400 ml), then $CHCl_3$ (400 ml).[29] Final $CHCl_3$ extract was colourless. Filtrates were pooled, concentrated under reduced pressure (to *ca.* 50 ml), then Et_2O (200 ml) was added. Organic layer was extracted with $NaHCO_3$ (5%, 50 ml × 4), then with H_2O (50 ml × 3). The residue was the neutral lipid fraction. $NaHCO_3$ solutions were acidified (pH 2) and extracted with EtOAc (50 ml × 5) Extracts were pooled, washed with HCl (0·01%, 50 ml × 2) and H_2O (50 ml × 1). The residue was the acid fraction.

Extractions of ent-*kaurene* (II) *from barley*. Dry decorticated barley (10 kg) was finely ground in an EBC Mill (Casella, London 0·5 mm screen). Portions (1 kg), in a glass column, were percolated with Me_2CO until the extract was colourless. The residue (rotary evaporation) was dissolved in Et_2O (1 l.), and washed (H_2O, 500 ml × 3). *ent*-Kaurene-[^{14}C] (75 000 dpm, 2·3 μg) was added to the lipid (182 g). The lipid was saponified. Hydrocarbons were separated from the non-saponifiable fraction (13·45 g) by alumina column chromatography (180 g, 20 × 3·5 cm; light petrol., b.p. 60–80°). All the radioactive material and 1·185 g of lipid was eluted in the first three fractions (250 ml). This material was rechromatographed (alumina column, 80 g, 25 × 2 cm, same solvent). The radioactive material was eluted in fractions 13–25 (10 ml each), which were pooled (19·3 mg). This material was used for the identification of *ent*-kaurene by GLC.

Identification of ent-*kaurene as* ent-*kauran*-16-*ol acetate*-[^{3}H]. Finely ground barley (200 g) was extracted in turn with iso-PrOH (800 ml), iso-PrOH–$CHCl_3$ (1:1; 800 ml), and $CHCl_3$ (800 ml). *ent*-Kaurene-[^{14}C] (22 500 dpm, 75 ng) was added. Lipid was recovered, dissolved in Et_2O and washed. The lipid was chromatographed 2× on silica-gel columns irrigated with light petrol. b.p. 40–60°, (100 g, 16 × 3·5 cm; first recovery 0·488 g; second recovery 0·2051 g, 19 800 dpm) then on an alumina column irrigated with hexane (50 g, 20 × 3·5 cm; recovery 12·8 mg (6650 dpm). Material stood with *m*-chloroperbenzoic acid (40 mg) in $CHCl_3$ (3 ml) overnight, at room temp. Solution was diluted with $CHCl_3$ (10 ml) and sequentially extracted with $FeSO_4$ (2%, 5 ml × 3), H_2O (5 ml × 3), $NaHCO_3$ (2%, 5 ml × 3), and H_2O (4 ml × 3). Product was recovered, dissolved in C_6H_6 (2 ml) and Red-al (1 ml, 70% sodium dihydro-bis-(2-methoxy-2-ethoxy) aluminate in C_6H_6), left 16 hr, then washed with H_2SO_4 (10%) and H_2O. The product *ent*-kauran-16-ol was purified by TLC, (solvent 4, recovery 3650 dpm), dissolved in anhyd. C_6H_6 (2 ml) with pyridine (0·1 ml) and acetic anhydride-[^{1}H] (1 mCi; sp. act. 100 μCi/μmol) and left at room temp. for 5 days. Calculations on the final product indicate that acetylation was *ca.* 60% efficient. EtOAc (2 ml) was added and the solution was washed with HCl (2 M, 1 ml × 3). The acetate zone was purified (TLC, solvent 4, recovery 7 430 000 dpm). Authentic unlabelled *ent*-kauran-16-ol acetate (50 mg previously prepared from *ent*-kaurene,[4,30] m.p. 212°, reported 212–214°,[4]) was added. Recrystallizations were from light petrol. The activity of the [^{3}H] was determined by scintillation counting.

Estimation of ent-*kaurene* (II). Barley samples (100 g) were germinated and lipids were extracted. Trace amounts of *ent*-kaurene-[^{14}C] (*ca.* 10 000 dpm, 35 ng) internal standard was added to the crude extract. The amount added was too small to be detected by GLC. The neutral lipid fraction was loaded on a silica gel column (100 g, 60–120 mesh, 17 × 3·5 cm), which was washed with hexane (250 ml). The residual hydrocarbon sample was separated by TLC (solvent 3). The radioactive band was recovered and the *ent*-kaurene (II) was converted into *ent*-kaurene norketone (IV). Typically 35% of the radioactivity was found in the purified *ent*-kaurene norketone before GLC. To each sample nonadecane (*ca.* 40 μg, accurately known) was added as a GLC internal standard. Aliquots were radioassayed. The remainder was dissolved in $CHCl_3$ (0·1 ml). Aliquots (10 μl) were separated by GLC (2·75 m column, 5% SE30, 197°, N_2 flow rate 50 ml/min). *ent*-Kaurene norketone, R_t 21 min; nonadecane, R_t 7 min.

TLC. Silica gel G plates were always used. R_fs are approximate, they varied considerably. Solvent 1, Hexane–EtOAc (7:3), *ent*-kaurene 0·9, *ent*-kauran-16,17-diol 0·45. Solvent 2, C_6H_6–EtOAc (19:1), *ent*-kaurene 0·9, *ent*-kaurene norketone 0·8, *ent*-kauran-16,17-epoxide 0·8, *ent*-kauran-16,17-diol, 0·02. Solvent 3, Hexane. *ent*-Kaurene 0·65, *ent*-17-norkaurane 0·7, *ent*-kauran-16-17-epoxide, 0. Solvent 4, C_6H_6–EtOAc (9:1), *ent*-kaurene 0·9, *ent*-kaurenal 0·85, *ent*-kauran-16,17-epoxide (Compound *C*) 0·85, *ent*-kaurene norketone 0·85, *ent*-kauran-16,17-diol diacetate 0·65, *ent*-kaurenol or *ent*-kauran-16- or 17-ol (Compound *B*) 0·45, acetates of *ent*-kaurenol, *ent*-kauran-16- or -17-ol 0·8, *ent*-kaurenoic acid or *ent*-kauran-17-oic acid 0·15, methyl esters of *ent*-kaurenoic and *ent*-kauranoic acids 0·80, *ent*-kauran-16,17-diol (Compound *A*) 0·07, 7-hydroxykaurenolide 0 12, *ent*-16-hydroxy-17-norkaurane 0·45. Solvent 5, EtOAc, *ent*-kaurene 1. Solvent 6, $CHCl_3$–EtOAc–HOAc (12:8·1), *ent*-kaurene 1, GA_3 0·17. Solvent 7, Hexane–EtOAc (4:1), *ent*-kaurene 1, *ent*-kaurenal 0·62, *ent*-kaurenoic acid 0·20, *ent*-kauran-16,17-epoxide 0·65. Solvent 8, C_6H_6–Hexane (1:1), double development- 4-(4′nitrophenylazo) benzoyl esters of *ent*-kaurenol, *ent*-kauran-16- or 17-ols 0·41. Solvent 9, C_6H_6–EtOAc (2:1), *ent*-kauran-16,17-diol 0·28, 16 methoxykauranol 0·45, *ent*-kauran-16- or -17-ol 0·67.

GLC. Carried out in a Pye 104 dual-column equipment, with F.I.D., using all-glass columns and N_2

[29] HALL, G. S. and LAIDMAN, D. L. (1968) *Biochem. J.* **108,** 475.
[30] HANSON, J. R. (1963) *J. Chem. Soc.* 5061.

carrier gas. Stationary phases were supported on acid-washed celite, 80–120 mesh. Identification of *ent*-kaurene (II) by GLC (i) 1·5 m column, 5% SE-30, N_2 80 ml/min, R_t 18 min at 170°, 15·9 min at 180°; (ii) 1·5 m column, 5% PEGA, 175°, N_2 80 ml/min, R_t 16 min; (iii) 1·5 m column, 5% QF1, N_2 80 ml/min, R_t 15 min at 135°, 7·5 min at 155°. GLC of *ent*-kauran-16,17-diol (III) (i) 1·5 m column 5% SE30, 210°, N_2 80 ml/min, R_t 8 min; (ii) 2·75 m column, 5% SE30, 220°, N_2 50 ml/min, R_t 20·8 min. GLC of *ent*-kaurene norketone (IV) (i) 0·92 m column, 5% QF1, 200°, N_2 40 ml/min, R_t 5·9 min; (ii) 2·75 m column, 5% SE30, 197°, N_2 50 ml/min, R_t 24·9 min. GLC of *ent*-17-norkaurane (V) (i) 1·5 m column, 5% OV101, 196°, N_2 60 ml/min, R_t 8·6 min; (ii) 2·75 m column, 5% SE30, 185°, N_2 50 ml/min, R_t 22 min.

Measurement of radioactivity Counting was in a Nuclear Chicago model 120 The scintillation fluid was 2,5-diphenyloxazole (5·0 g) and 1,4-di-2-(5-phenylaxazole)-benzene (0·1 g) in toluene (1 l). Initially chromatograms were examined using a Packard Model 7201 radiochromatogram scanner.

Materials. GA_3, was obtained from Plant Protection Ltd. *Gibberella* mycelium, *ent*-kaurene and *ent*-kaurenoic acid were gifts. 4-(4'Nitrophenylazo) benzoyl chloride was synthesized.[31]

ent-*Kaurene*-[^{14}C] *Gibberella fujikuroi* was grown,[32] and the mycelial pad from 1 l. culture was disintegrated in buffer (30 ml, 0·1 M phosphate pH 7·4, $MgCl_2$ 12 mM, mercaptoethanol 5 mM), with glass ballotini beads (0·25 mm, 50 g) in a Braun MSK 2876 homogenizer. A microsome-free supernatant, prepared as for barley, had a protein content of 4–6 mg/ml. Supernatant (5 ml) was incubated with ATP (60 μmol) and mevalonic acid-[2-^{14}C] (2 μmol, 20 μCi; 3 hr, 30°). These conditions were found to be optimal. The reaction termination and lipid extraction were as for barley cell-free preparations. The *ent*-kaurene-[^{14}C] was purified by TLC (solvent 3).

7-*Hydroxy kaurenolide.* (*ent*-6β-Hydroxy-7α-hydroxykaur-16-en-19-oic acid-19→6β lactone) was separated from the neutral and basic lipid fraction of a mycelial mat of *Gibberella fujikuroi* from an industrial fermentation. The oil was fractionated on an alumina column (35 × 7 cm) irrigated with 20% diethyl ether in light petrol. (2·1) The eluate was fractionated on an alumina column irrigated with light petrol. containing EtOAc, increasing by 5% in 1 l. steps. Material which separated from the 15–20% EtOAc fraction was purified by TLC, and crystallized as short thick rods (TLC, silica gel G, C_6H_6–EtOAc 7 3, R_f *ca.* 0·5; preparative TLC, solvent 4, R_f 0 12) m p 188° (reported m.p. 187–188°)[33] m p acetate 185° (reported m.p. 182–184°).[33]

ent-*Kaurenoic acid* (VIII) Prepared from the 7-hydroxykaurenolide[34] m p. 179–180° (reported m.p. 165–167°,[34] and 179–181°).[35] Methyl ester, m.p 71–72° (reported m.p. 71–72°).[34] The methylated product had the same retention time as authentic *ent*-kaurenoic acid methyl ester on GLC (0 92 m column, 5% QF1, 150°, N_2 flow rate 44 ml/min, R_t 11·5 min).

ent-*Kaurenol* (VII). *ent*-Kaurenoic acid (VIII) was methylated with CH_2N_2. *ent*-Kaurenoic acid methyl ester (48 mg free acid) was reduced with sodium dihydro bis-(2-methoxy-2-ethoxy) aluminate (350 mg) in dry C_6H_6 (15 ml). added in drops. The mixture was refluxed 1 hr, cooled, washed with H_2SO_4 (10%; 20 ml × 2), then H_2O. Product was purified by TLC (solvent 4), yield 90% based on the acid. Recrystallized from light petrol m.p. 140–142° (reported 141–142°).[35]

ent-*Kaurenal* (IX). *ent*-Kaurenol (VII; 10 mg) in anydrous pyridine (0·1 ml) oxidised at room temp by the addition, 1 hr apart, of 2 samples of chromic anhydride-pyridine complex (0·1 ml).[17,36] After 2 hr MeOH (1 0 ml) was added followed by H_2O (5 ml). Products were extracted into EtOAc and purified by TLC (solvent 4)

ent-*Kauran*-16,17-epoxide (X). The method was originally for squalene-2,3-epoxide [37] *ent*-Kaurene (II; 10 mg) was dissolved in tetrahydrofuran (10·5 ml) and H_2O (10 ml). N-Bromosuccinimide (10 mg) was added and the solution stirrer (under N_2, 30 min, 0°) Product was extracted into hexane. Residue from hexane was dissolved in MeOH (10 ml) and K_2CO_3 (0 5 g) was added, then the suspension was stirred (room temp., 3 hr under N_2) The epoxide was recovered then purified by TLC (first solvent 3, to remove unreacted *ent*-kaurene, then solvent 2).

ent-*Kauran*-16-*ol* (VI). *ent*-Kauran-16-17 epoxide (X), compound *C*, was reduced with sodium dihydro-bis-(2-methoxy-2-ethoxy) aluminate to give *ent*-kauran-16-ol.[30] Manipulations were as for the reduction of *ent*-kaurenoic acid methyl ester.

4-(4'-*Nitrophenylazo*) *benzoyl esters* of alcohols were prepared and purified by the technique previously used for terpenols.[2,31]

[31] HECKER, E. (1955) *Chem. Ber.* **88,** 1666.
[32] SHECHTER, I. and WEST, C. A. (1969) *J. Biol. Chem.* **244,** 3200.
[33] CROSS, B. E., GALT, R. H. B., HANSON, J. R., CURTIS, P. J., GROVE, J. F. MORRISON, A. (1963) *J. Chem. Soc.* 2937.
[34] GALT, R. H. B. and HANSON, J. R. (1966) *Tetrahedron* **22,** 3185.
[35] HENRICK, C. A. and JEFFERIES, P R. (1964) *Australian J. Chem.* **17,** 915.
[36] POOS, G. I., ARTH, G. E., BEYLER, R E. and SARETT, L. H. (1953) *J. Am. Chem. Soc.* **75,** 427.
[37] NADEAU, R. G. and HANZLIK, R. P. (1969) in *Methods in Enzymology* (CLAYTON, R. B., ed.), Vol. 15, p. 346, Academic Press, New York.

ent-*Kauran*-16,17-*diol* (III), *ent*-Kaurene (II; 25 mg) was dissolved in pyridine (5 ml). OsO_4 (20 mg) was added to the stirred solution, which was left *ca.* 18 hr room temp. H_2O (15 ml), pyridine (12·5 ml) and $NaHSO_3$ (100 mg) were added and the mixture was stirred 30 min, then poured into EtOAc (200 ml). Solution washed with HCl (0·1 M, 100 ml × 3) then H_2O. Product was purified by TLC (solvent 2) and re-crystallized from Me_2CO. The m.p. was 191° (reported m.p. 189°).[4]

ent-*Kauran*-16,17-*diol diacetate*. *ent*-Kauran-16,17-diol (III) was acetylated with $(MeCO)_2O$ in pyridine (1:1), 5 days, room temp. The mixture was dissolved in EtOAc, and washed with dil. HCl and H_2O before recovery.

ent-*Kaurene norketone* (ent-16-*Oxo*-17-*norkaurane*; IV). *ent*-Kauran-16,17-diol (III) (30 mg) was dissolved in tetrahydrofuran (10 ml). $NaIO_4$ (50 mg) was added in H_2O (5 ml) to the stirred solution which was left *ca.* 18 hr room temp. HCl (0·1 ml, 2 M) was added and the mixture was poured into EtOAc (25 ml). This was washed with H_2O (10 ml × 3) then dried. The norketone was purified by TLC (solvent 2) and recrystallized from H_2O–MeOH m.p. 117° (reported m.p. 117°).[4]

ent-16-*Hydroxy*-17-*norkaurane* (XIV). Reduction of *ent*-kaurene norketone (IV) with $NaBH_4$ in MeOH (2 hr room temp.) gave material moving in the expected region on TLC (solvent 4).[19]

ent-17-*Norkaurane* (V) (*Wolff–Kishner reduction*). *ent*-Kaurene norketone (IV) (30 mg) to diglyme (5 ml) and hydrazine hydrate (0·1 ml) heated at 150°, 2 hr, KOH (300 mg) was added and the temp. was raised to 250° for 4 hr. The solution was cooled then poured into H_2O, acidified and extracted with EtOAc. The crude product was purified by TLC (solvent 3).

Chemical modifications of ent-*kaurene*-[^{14}C]-*derivatives*. *Acid treatment*. In attempts to hydrate 16,17-double bonds, compounds were dissolved in H_2O (4 ml) with MeOH (1 ml). Conc. HCl was added (1·25 ml). The mixture was stored 3 days at room temp.[38] Products were extracted with EtOAc. Some product formation from addition of MeOH also occurred. *Oxidation*. (1) *Aldehydes with alkaline* $AgNO_3$. The compound was dissolved in EtOH (2 ml) and stirred as $AgNO_3$ (18·5 mg) in H_2O (0·1 ml) was added, followed by NaOH (0·5 ml, 0·5 M).[39] Mixture was stirred *ca.* 18 hr then HCl (1·0 ml. 1·0 M) and H_2O (10 ml) were added. The product was extracted with EtOAc. (2) *Oxidation of alcohols with Jones' reagent*. The compound was dissolved in Me_2CO (1·0 ml) and Jones reagent (0·1 ml) was added.[40] After 1 hr H_2O (5 ml) was added. Products were extracted into EtOAc.

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[38] Cross, B. E., Galt, R. H. B. and Hanson, J. R. (1962) *Tetrahedron* **18**, 451.
[39] Sheehan, J. C. and Robinson, C. A. (1951) *J. Am. Chem. Soc.* **73**, 1207.
[40] Curtis, R. G., Heilbron, I. Jones, E. R. H. and Woods, G. F. (1953) *J. Chem. Soc.* 457