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METABOLISM OF *ENT*-KAURENOL-[17-^{14}C], *ENT*-KAURENAL-[17-^{14}C] AND *ENT*-KAURENOIC ACID-[17-^{14}C] BY GERMINATING *HORDEUM DISTICHON* GRAINS*

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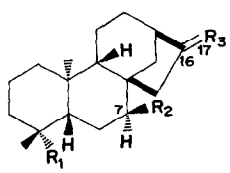
Key Word Index—*Hordeum distichon*; Gramineae; barley; metabolism; *ent*-kaurenol; *ent*-kaurenal; *ent*-kaurenoic acid; *ent*-hydroxykaurenoic acid; gibberellins.

Abstract—Subcellular fractions from germinated barley embryos, chloroplast preparations and whole germinating barley grains are able to carry out the conversions *ent*-kaurenol → *ent*-kaurenal → *ent*-kaurenoic acid → *ent*-hydroxykaurenoic acid, the initial steps of the biosynthetic pathway to gibberellins. Whole grains, and chloroplasts to a slight extent, incorporate radioactivity from *ent*-kaurenol-[17-^{14}C] and *ent*-kaurenoic acid-[17-^{14}C] into materials with similar but distinct properties from the gibberellins GA_1, GA_3, GA_4 and GA_7.

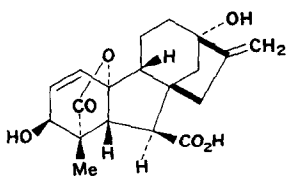
INTRODUCTION

ent-Kaurene (**1**), *ent*-kaurenol (**2**), *ent*-kaurenal (**3**), *ent*-kaurenoic acid (**4**) and *ent*-hydroxykaurenoic acid (**5**) are gibberellin precursors in *Gibberella fujikuroi* [1–6], and are metabolized by various higher plants [1,2,5,7–10]. However, only with *Cucurbita pepo* has their incorporation into a fully characterized gibberellin been demonstrated [8].

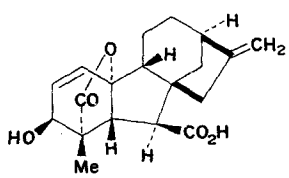


	R_1	R_2	R_3
(1)	– Me	–H	$=CH_2$
(2)	– CH_2OH	–H	$=CH_2$
(3)	– CHO	–H	$=CH_2$
(4)	– CO_2H	–H	$=CH_2$
(5)	– CO_2H	–OH	$=CH_2$
(8)	– CO_2H	–H	–H,–OH

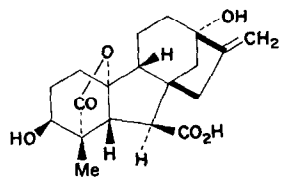
We have detected GA_3 (**6**) and GA_7 (**7**) in barley, but could find no incorporation of mevalonic acid-[2-^{14}C] into *ent*-kaurene (**1**) or GA_3 (**6**) [11]. *ent*-Kaurene (**1**) occurs in barley, and it probably acts as a stored precursor for gibberellin synthesis [12]. Despite this, whole barley did not metabolize exogenous *ent*-kaurene-[17-^{14}C] and embryo extracts converted it, via enzymatic and non-enzymatic processes, into *ent*-kauran-17-ol, *ent*-kauran-16,17-epoxide and *ent*-kauran-16,17-diol, substances not on the biosynthetic pathway to gibberellins [12].



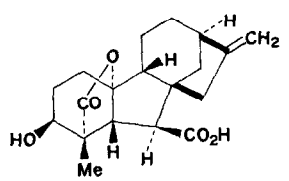
GA_3 (6)



GA_7 (7)



GA_1 (9)



GA_4 (10)

* Trivial names are used as follows: *ent*-kaurene = *ent*-kaur-16-ene (**1**); *ent*-kaurenol = *ent*-kaur-16-en-19-ol (**2**); *ent*-kaurenal = *ent*-kaur-16-en-19-al (**3**); *ent*-kaurenoic acid = *ent*-kaur-16-en-19-oic acid (**4**); *ent*-hydroxykaurenoic acid = *ent*-7α-hydroxy-kaur-16-en-19-oic acid (**5**).

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RESULTS

Metabolism of ent-*kaurenol* (**2**), ent-*kaurenal* (**3**) *and* ent-*kaurenoic acid* (**4**) *by barley preparations*

Various washed particulate fractions were prepared from separated barley embryos. These fractions were incubated with radioactive *ent*-kaurenol, *ent*-kaurenal and *ent*-kaurenoic acid, all-[17-^{14}C], and the products were separated (see Experimental). The *ent*-kaurenol (**2**) was converted into *ent*-kaurenal (**3**) which in turn was changed into *ent*-kaurenoic acid (**4**). Typical figures for an experiment with a microsomal preparation are shown in Table 1(a). The reaction sequence occurred in each of the particulate fractions although in terms of the activity/mg protein the activity was highest in the microsomal fraction.

ent-Kaurenoic acid-[17-^{14}C] was incubated with the particulate fractions and the products were separated by TLC (solvents 1 and 2). In each case the same reaction products were found. A more polar material with the same mobility as authentic *ent*-hydroxykaurenoic acid (**5**) and *ent*-16-hydroxy-17-norkaurenoic acid (**8**) was obtained in 1·5% yield with the microsomal fraction. After methylation or methylation and acetylation the radioactive material moved with the derivatives of *ent*-16-hydroxy-17-norkaurenoic acid (**8**). Material purified by TLC (solvent 1) from incubations with mitochondria or microsomes was added to authentic *ent*-hydroxykaurenoic acid (**5**; 20 mg) and recrystallized to constant specific activity (microsomes 42 dpm/mg, 0·36% incorporation; mitochondria 25 dpm/mg, 0·22% incorporation). Thus *ent*-hydroxykaurenoic acid (**5**) was present in each sample.

So microsomal and other preparations will successively convert *ent*-kaurenol (**2**) to *ent*-kaurenal (**3**), to *ent*-kaurenoic acid (**4**) to *ent*-hydroxykaurenoic acid (**5**). The first two conversions were not carried out by alcohol or aldehyde dehydrogenases as these could not be detected in the microsomal fraction using either NAD^+ or $NADP^+$ as cofactor. In contrast the supernatant fraction was rich in ethanol dehydrogenase ($\Delta E_{340}NAD^+$, 1·24/mg protein/min; $\Delta E_{340}NADP^+$, 0·0057/mg protein/min). Possibly mixed-function oxygenases were involved in the observed interconversions [13]. *ent*-Kaurenol-[17-^{14}C] and *ent*-kaurenal-[17-^{14}C] were incubated with microsomal preparations in the presence of various cofactors with air or carbon monoxide as the gas phase. The respective conversions were determined on the separated products (TLC solvent 1). When the formation of product without added cofactor = 100, then for *ent*-kaurenal (**3**) synthesis: $+NADP^+$ = 135; NADPH = 222; NADPH + CO = 184; and for *ent*-kaurenoic acid (**4**) synthesis: $+NADP^+$ = 98; NADPH = 125; NADPH + CO = 43. The type of result was reproducible.

As chloroplasts from kale convert *ent*-kaurenoic acid (**4**) into gibberellin-like materials [10], chloroplasts from young barley leaves were incubated with *ent*-kaurenol-[17-^{14}C] and *ent*-kaurenoic acid-[17-^{14}C]. Estimation of the products (see Experimental) showed that 17% of the *ent*-kaurenol (**2**) was converted into *ent*-kaurenal (**3**). *ent*-Kaurenoic acid (**4**) was converted to more polar materials (TLC solvent 3). One of these behaved like the derivatives of *ent*-hydroxykaurenoic acid (**5**) following methylation or methylation and acetylation. A portion of this material was added to authentic *ent*-hydroxykaurenoic acid (**5**; 24 mg) and recrystallized to a constant specific radioactivity (24 dpm/mg), showing that *ent*-hydroxykaurenoic acid (**5**) had been formed. Other

Table 1. Characterization of the products of the metabolism of *ent*-kaurenol and *ent*-kaurenal

Substrate	Suspected product	Recovery as *ent*-kaurenol (dpm)	Amount of carrier added (mg)	Ester (mg)	Recovered (dpm/mg)	Final (mg)	Recovery (dpm/mg)
(a) *ent*-Kaurenol	*ent*-Kaurenal	2850	5·6	7·2	245	1·62	270
ent-Kaurenal	*ent*-Kaurenoic acid	1118	7·4	8·35	69	1·72	78
(b) *ent*-Kaurenol	*ent*-Kaurenal	410	5·6	3·62	26	0·80	30·2
ent-Kaurenal	*ent*-Kaurenoic acid	450	8·7	8·33	27	0·77	30·5

ent-Kaurenol or *ent*-kaurenal were incubated with a microsomal preparation from germinating barley (a) or whole barley (b). Products initially chromatographing with *ent*-kaurenal or *ent*-kaurenoic acid were recovered, converted to *ent*-kaurenol, added to carrier, and the 4-(4′-nitrophenylazo)-benzoyl esters made and recrystallized.

polar products from *ent*-kaurenoic acid (**4**) behaved like the gibberellin pairs GA_1/GA_3 (**9**, **6**) and GA_4/GA_7 (**10**, **7**) on TLC (solvent 4). Treatment of eluted materials with dilute acid followed by TLC (solvent 3) showed no radioactivity in the zones occupied by the authentic gibberellin degradation products [11,14]. Thus these barley chloroplast preparations did not make GA_3 (**6**), GA_1 (**9**), GA_4 (**10**), or GA_7 (**7**) from *ent*-kaurenoic acid (**4**).

Metabolism of ent-*kaurenol*-[17-^{14}C] *and* ent-*kaurenoic acid*-[17-^{14}C] *by germinating barley grains*

The non-saponifiable part of the neutral lipid fraction from barley incubated with *ent*-kaurenol-[17-^{14}C] contained 90·5% of the original radioactivity. This fraction contained materials chromatographing with *ent*-kaurenal (**3**) and *ent*-kaurenoic acid (**4**). The identity of these materials was confirmed (Table 1b).

The lipid from barley germinated with *ent*-kaurenoic acid-[17-^{14}C] was partitioned between ether and NaOH (5%) but only 25% of the radioactivity was extracted into the aqueous phase [15,16]. When part of the NaOH-insoluble lipid was chromatographed (TLC, solvent 1, double development) most of the radioactivity moved with *ent*-kaurenoic acid (**4**).

The material extracted with NaOH was recovered and methylated. On TLC (solvent 1) a material moving with *ent*-hydroxykaurenoic acid methyl ester, was obtained in small amounts. Following acetylation of the methyl esters, radioactivity moved with the marker *ent*-hydroxykaurenoic acid derivative (TLC, solvent 1). Portions of the radioactive material were demethylated and added to either *ent*-7α-hydroxy-kaur-16-en-19-oic acid (**5**; 25 mg) or to *ent*-7β-hydroxy-kaur-16-en-19-oic acid (40 mg). On recrystallization radioactivity (6 dpm/mg) was only retained in the crystals of **5**, the known gibberellin precursor.

The acidic lipid fraction, from barley germinated in the presence of *ent*-kaurenol-[17-^{14}C], contained 2·4% of the applied radioactivity, while that from incubation with *ent*-kaurenoic acid-[17-^{14}C] contained 6·0%. In each case TLC (solvent 4) of the acid fraction showed radioactivity in the GA_1/GA_3 (**9**, **6**) and GA_4/GA_7 (**10**, **7**) zones as well as other areas. (These pairs of gibberellins and some of their derivatives are difficult to separate.)

Samples (450 dpm) were methylated then chromatographed (TLC, solvent 6) or acetylated and chromatographed (TLC, solvent 3) when radioactivity moved with the authentic derivatives of GA_1/GA_3. GA_3 (**6**; 100 mg) was added to a sample (2270 dpm) from the GA_1/GA_3 zone, the mixture was methylated and the purified esters (TLC, solvent 6) were recrystallized. Radioactivity was lost so the radioactive material was not GA_3 (**6**). Treatment of the material with dilute acid gave a product moving close to the GA_1 degradation product (TLC, solvent 3), and distinct from the GA_3 degradation product. The original material (11 740 dpm) was added to GA_1 (**9**; 50 mg), the mixture was methylated and separated by TLC (solvent 6). The recovered methyl esters were recrystallized, when the specific activity declined to a low level (4th crystals, 1·0 dpm/mg), so it was improbable that GA_1-[^{14}C] was originally present.

Radioactive material from the GA_4/GA_7 (**10**, **7**) zone, or its derivatives, moved with GA_7 (**7**; TLC, solvent 7), GA_7 acetate (TLC, solvent 3) and GA_7 methyl ester (TLC, solvent 8). Following acid treatment of the material radioactivity remained near the baseline (TLC, solvent 3), close to one of the breakdown products of GA_4 and distinct from the product of GA_7. Samples (6570 dpm) from the GA_4/GA_7 (**10**, **7**) zone were added to authentic GA_4 (**10**; 40 mg) or GA_7 (**7**; 40 mg) and were methylated. In each case, following purification by TLC (solvent 9) recrystallization reduced the specific activity to very low levels (4th crystals GA_4 methyl ester *ca* 1 dpm/mg; GA_7 methyl ester *ca* 0·4 dpm/mg). Thus little or no radioactive GA_4 (**10**) or GA_7 (**7**) could have been present.

The experiment was repeated with two equal applications of *ent*-kaurenoic acid-[17-^{14}C] (2 330 000 dpm) to barley (200 g) after steeping and after 12 hr germination. Carrier GA_1 (**9**), GA_3 (**6**), GA_4 (**10**) and GA_7 (**7**) (100 mg each) were added to the lipid extract and the GA_1/GA_3 and GA_4/GA_7 fractions were separated from the acidic materials by preparative TLC (solvent 3). The individual gibberellins were separated on partition columns and crystallized. Recrystallization (× 3) reduced the specific radioactivities to very low values (1–3 dpm/mg) which declined more on further recrystallizations. Thus significant incorporation of *ent*-kaurenoic acid (**4**) into these gibberellins had not occurred.

DISCUSSION

Germinating barley does not incorporate added mevalonic acid-[2-^{14}C] into *ent*-kaurene or gibberellins [11], although endogenous gibberellins increase in amount [17], and GA_3 and GA_7 are present [11]. These observations, the occurrence of endogenous *ent*-kaurene, and its decline in the early stages of germination led us to suggest that it acts as a stored precursor of barley gibberellins [12]. However, with embryo homogenates *ent*-kaurene is converted to products which are not recognized gibberellin precursors [12].

The present demonstration of the sequential conversion of the more hydrophilic *ent*-kaurenol through *ent*-kaurenal and *ent*-kaurenoic acid to *ent*-hydroxykaurenoic acid shows that the recognized initial stages of gibberellin synthesis are occurring [1,2,4,7,8]. The further metabolism into unidentified products that are chromatographically and chemically similar to, but distinct from, the gibberellins GA_1, GA_3, GA_4 and GA_7 may be due to the formation of by-products, gibberellin precursors or other gibberellins. Clarification must await further studies, as must the reason for the failure to obtain incorporation of *ent*-kaurenol and *ent*-kaurenoic acid into the GA_3 and GA_7 which are present [11]. Possibly the synthesis of gibberellins required for germination occurs before the added precursors reach the synthetic site. Alternatively, the exogenous precursors may not reach the site of gibberellin synthesis, and may be metabolized by an alternative catabolic pathway.

EXPERIMENTAL

Barley preparations. Barley grains, *Hordeum distichon* cv. Proctor, were selected, decorticated, sterilized, and germinated under malting conditions [11,17]. Cell-free particulate preparations were prepared from isolated embryos, as before [12], from grain germinated 38 hr at 14·4°, and homogenized in sucrose (0·25 M) chloroplast medium [10]. Washed particulate preparations sedimenting at 1000 g/20 min (cell debris), 12000 g/20 min (mitochondria) and 100000 g/1 hr (microsomes) were obtained and used suspended in another medium [18]. Chloroplasts were prepared from young first leaves of barley by Stoddart's method [10].

Gibberellins. GA_3 (**6**) was purchased from Plant Protection Ltd. GA_1 (**9**) was prepared by reduction of GA_3 (**6**) [19] and purified by chromatography [20]. GA_4 (**10**) and GA_7 (**7**) were separated by chromatography [20] from a mixture received as a gift. Acid degradation (10% HCl, 55°, 2 hr), methylations and acetylations were carried out as before [11,12].

ent-16-*Hydroxy*-17-*norkauren*-19-*oic acid* (**8**). *ent*-Kaurenoic acid (**4**) was converted to the equivalent norketone (*ent*-16-oxo-17-norkauren-19-oic acid) as described for *ent*-kaurene norketone [12]. The product was reduced with $NaBH_4$ in MeOH (2 hr, room temp.), and was purified by TLC (solvent 3).

ent-*Hydroxykaurenoic acid* (**5**). 7-Oxo-kaurenoic acid, prepared from 7-hydroxy-kaurenolide [21] was reduced to a mixture of 7α- and 7β-hydroxykaurenoic acids [22] which were separated by TLC (solvent 11). Owing to the extreme insolubility of the product only 50 mg could be purified on one 0·5 mm thick plate. Recrystallization of the purified material from EtOAc–light petrol. (b.p. 60–80°) gave 210 mg of *ent*-7α-hydroxykaurenoic acid (overall yield from 7-hydroxykaurenolide 42%) m.p. 248–252° (reported 255–258°) [22]; methyl ester m.p. 177–182° (reported 182–184°) [22]. *ent*-7β-Hydroxykaurenoic acid was obtained in overall yield of 26%, m.p. 245–248° (reported 248–250°) [22].

ent-*Kaurenoic acid*-[17-^{14}C]. Unlabelled *ent*-kaurenoic acid (**4**) was converted to *ent*-16-oxo-17-norkaurenoic acid (*ent*-kaurenoic acid norketone) [12]. MeI-[^{14}C] (24·9 mg, 88 μCi) was treated (room temp., 3 days) with triphenyl phosphine (60 mg) in dry C_6H_6 (8 ml). The precipitated product (59·6 mg) was suspended in dry tetrahydrofuran (10 ml) under N_2, butyl lithium (0·5 ml, 1·5 M in hexane) was added, followed by *ent*-kaurenoic acid norketone (34 mg) in tetrahydrofuran (2·0 ml). The solution, maintained under N_2, was stirred overnight, refluxed 5 hr, cooled, and poured into HCl (0·1 M, 25 ml). The product was extracted into Et_2O, washed with HCl (0·1 M, 25 ml × 2), then H_2O (25 ml × 3) and finally purified by TLC (solvent 10). Yield 11·8 mg, 19·5 μCi.

Product and substrate manipulation. Recoveries of lipids, determinations of radioactivity, addition of water to double bonds, and the preparation and interconversion of *ent*-kaurenol, *ent*-kaurenal and *ent*-kaurenoic acid were carried out as before [11,12]. Melting points, determined on a Koffler hot-stage apparatus, were uncorrected. In experiments investigating the conversions of *ent*-kaurenol (**2**) to *ent*-kaurenal (**3**) and this to *ent*-kaurenoic acid (**4**) the products, separated by TLC, were characterized by reducing them to *ent*-kaurenol, purifying this by TLC (solvent 1), adding authentic carrier, then preparing and recrystallizing the purified 4-(4′-nitrophenylazo) benzoyl ester to constant specific activity [11,12].

Recrystallization of radioactive products. Recrystallizations were performed until three successive specific radioactivities agreed within reasonable experimental error. The following solvents were used: (a) EtAc–light petrol. (b.p. 60–80°): methyl esters of GA_1, GA_4, GA_7, the gibberellins GA_1, GA_3, GA_4, GA_7 and *ent*-hydroxykaurenoic acid; (b) C_6H_6–MeOH–GA_3 methyl ester; (c) C_6H_6: 4-(4-nitrophenylazo) benzoyl esters.

Alcohol and aldehyde dehydrogenases. Were assayed according to Bonnichsen and Brink [23] and Black [24].

Partition chromatography of the gibberellins. The TLC (solvent 3) gibberellin fractions from the second *ent*-kaurenoic acid-[17-^{14}C] experiment were separated on partition columns of Sephadex G25 [20]. The GA_1 + GA_3 fraction (216·9 mg; 17260 dpm) was separated using C_6H_6–EtOAc–HOAc–H_2O (11:5:6:10) as solvent, while the GA_4 + GA_7 fraction (251·8 mg, 9065 dpm) was purified using C_6H_6–light petrol. (b.p. 60–80°)–EtOAc–H_2O (6:2:5:3). Fractions (15 ml) were collected and assayed for gibberellin content by taking 0·1 ml, drying, dissolving in H_2O (5 ml), adding $KMnO_4$ (0·5%, 0·1 ml) and reading the E_{380} against a $KMnO_4$ blank after 20 min. The indicated fractions were grouped and recrystallized (m.p.: GA_1, 250–255°; GA_3, 220–224°; GA_4, 215–220°; GA_7, 170–175°).

Thin layer chromatography. TLC plates of silica gel G were used, of 0·5 mm thickness. Average R_fs are given for the following solvents: (1) C_6H_6–EtOAc (9:1); *ent*-kaurenoic acid, 0·15;

ent-kaurenol, 0·48; *ent*-kaurenal, 0·85; *ent*-kaurenoic acid methyl ester, 0·85; *ent*-16-hydroxy-17-norkaurenoic acid methyl ester, 0·48; acetate of *ent*-16-hydroxy-17-norkaurenoic acid, 0·80. (2) C_6H_6–EtOAc (7:3): *ent*-16-hydroxy-17-norkaurenoic acid methyl ester, 0·5: hydroxylated *ent*-kaurenoic acid after acid treatment and methylation, 0·15. (3) Di-isopropyl ether–HOAc (19:1): *ent*-kaurenoic acid, 0·80; *ent*-hydroxykaurenoic acid, 0·54; *ent*-16-hydroxy-17-norkaurenoic acid, 0·54; GA_1 and GA_3, 0·4; GA_4 and GA_7, 0·68; acid degradation products, of GA_1, 0·08; GA_3, 0·33; GA_4, 0·22; GA_7, 0·34; 3-*O*-acetyl GA_1 or GA_3, 0·43; 3,13-di-*O*-acetyl GA_1 or GA_3, 0·65; 3-*O*-acetyl GA_4 or GA_7, 0·55. (4) $CHCl_3$–EtOAc–HOAc (12:8:1): GA_1 or GA_3, 0·15; GA_4 or GA_7, 0·35; *ent*-kaurenol or *ent*-kaurenoic acid, 0·90. (5) iso-PrOH–H_2O (4:1): GA_1 or GA_3, 0·60. (6) EtOAc-: GA_1 or GA_3 methyl ester, 0·5. (7) EtOAc–$CHCl_3$–HOAc (15:5:1): GA_4 or GA_7, 0·55. (8) C_6H_6–HOAc–H_2O (8:3:5; upper phase): GA_4 or GA_7 methyl ester, 0·45. (9) Hexane–EtOAc (1:1): GA_4 and GA_7 methyl ester, 0·45. (10) Hexane–EtOAc (4:1): *ent*-kaurenoic acid, 0·2. (11) EtOAc–light petrol. (b.p. 60–80°) (1:1): *ent*-hydroxykaurenoic acid, 0·42; *ent*-7β-hydroxykaurenoic acid, 0·32.

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