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THE FATE OF C-20 IN C_{19} GIBBERELLIN BIOSYNTHESIS*

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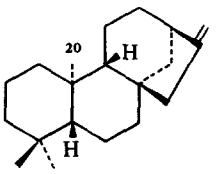
Key Word Index—*Gibberella fujikuroi*; biosynthesis; gibberellic acid; *ent*-kaur-16-ene.

Abstract—Experiments with *ent*-kaur-16-ene-[^{14}C], prepared biosynthetically from sodium acetate-[2-^{14}C], have shown that the C-20 carbon atom of the C_{20} gibberellins is evolved as carbon dioxide during the biosynthesis of the C_{19} gibberellins by *Gibberella fujikuroi*.

INTRODUCTION

The biosynthesis of the C_{19} plant hormone, gibberellic acid (**5**) involves the hydroxylation and oxidation of *ent*-kaur-16-ene (**1**) to *ent*-7α-hydroxy-kaur-16-en-19-oic acid (**2**) [1,2] the oxidative ring contraction of this to gibberellin A_{12} 7-aldehyde (**3**) [1–3], and the conversion of the latter via gibberellin A_4 (**4**) to gibberellic acid (**5**) [4–6]. An important stage in this biosynthesis involves the loss of the C-20 carbon atom and the formation of the 19 → 10-γ-lactone ring which occupies the same face of the molecule. In this paper we report our experiments concerning the final fate of the C-20 carbon atom. Our objective was to trap this carbon atom by feeding *ent*-kaur-16-ene (**1**) labelled at C-20 and, bearing in mind the examples of sterol demethylation [7–13], seeking the label in either formaldehyde, formic acid or carbon dioxide.



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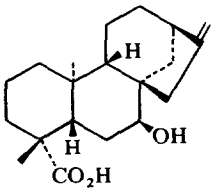

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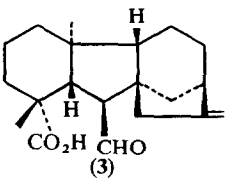
RESULTS AND DISCUSSION

The labelling pattern of kauranoid diterpenes from terpenoid precursors has been established [14,15]. In particular the 2- and 3'-mevalonoid labelling patterns show that C-20 is derived from C-2 of acetate. *ent*-Kaur-16-ene (**1**) labelled, *inter alia* at C-20, was obtained biosynthetically from sodium acetate-[2-^{14}C]. A series of incubations were carried out in order to optimize this labelling. Sodium acetate-[2-^{14}C] was fed to *Gibberella fujikuroi* ACC 917 for 12 and 24 hr periods on days 3, 4 and 5 of the fermentation. The optimum incorporation (see details in Experimental) was for a 12 hr incubation on day 4.



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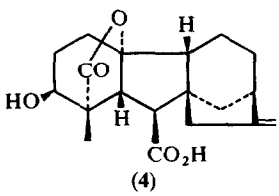
The fermentation produces substantial quantities of carbon dioxide. Hence the biosynthesis of endogenous *ent*-kaur-16-ene was inhibited by AMO-1618 in order to minimize dilution from this source. It has been shown [16] that AMO-1618 inhibits the cyclization stages in kaurene biosynthesis. When *ent*-kaur-16-ene-[^{14}C] was fed to a fermentation inhibited by 10^{-4} M AMO-1618, its incorporation into gibberellic acid was 31.6% and into gibberellin A_4/A_7, 12.6% (cf. normal fermentations *ca* 5%).



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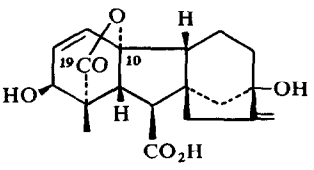

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* Part 21 of the series 'Studies in Terpenoid Biosynthesis'. For part 20 see Dockerill, B. and Hanson, J. R. (1978) *Phytochemistry* **17**, 427.

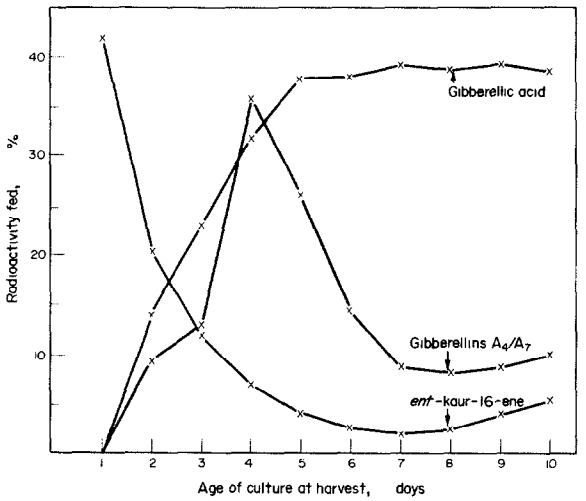


Fig. 1. Incorporation of *ent*-kaur-16-ene-[^{14}C] into gibberellic acid and gibberellins A_4/A_7. Cultures were fed *ent*-kaur-16-ene-[^{14}C] at inoculation

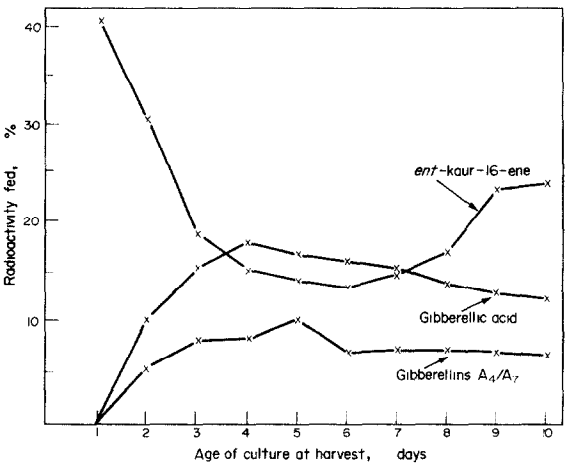


Fig. 2. Incorporation of *ent*-kaur-16-ene-[^{14}C] into gibberellic acid and gibberellins A_4/A_7. Cultures were fed *ent*-kaur-16-ene-[^{14}C] daily and harvested 24 hr later.

This greater incorporation is the result of less dilution by endogenous material. Apart from confirming the fact [16] that the post-kaurene stages of gibberellic acid biosynthesis are not inhibited by AMO-1618, this suggests a method for preparing gibberellic acid of high specific activity since ent-kaurene can be made from mevalonate by a cell-free system with relatively little dilution by endogenous material [15].

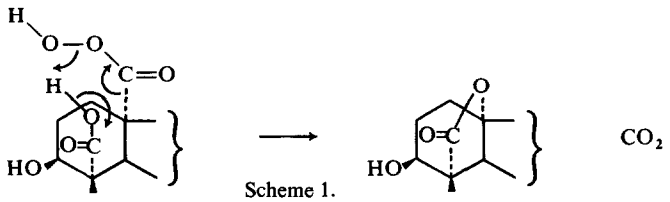
The optimum stage of the fermentation for feeding the *ent*-kaur-16-ene was then established by two experiments. In the first *ent*-kaur-16-ene-[^{14}C] was fed at the time of inoculation and then the *ent*-kaur-16-ene, gibberellins A_4/A_7 and gibberellic acid were recovered from the broth at daily intervals (Fig. 1). In the second experiment *ent*-kaur-16-ene-[^{14}C] was fed daily. The fermentations were harvested and the metabolites isolated 24 hr later (Fig. 2). In the light of these experiments further incubations used four-day old cultures of *G. fujikuroi* and an incubation period of 24 hr. The results indicated a maximum in gibberellic acid production at this stage. The slight upturn in recovered *ent*-kaur-16-ene-[^{14}C] towards the end of the fermentation (Fig. 1) is probably indicative of cell-bound material being released by cell lysis at this stage in the fermentation.

The incubations with *ent*-kaur-16-ene-[^{14}C] prepared biosynthetically from sodium acetate-[2-^{14}C] were then carried out. The AMO-1618 inhibited cultures were grown for four days, the *ent*-kaur-16-ene-[^{14}C] (**1**) was added and the flasks were sealed and incubated for 24 hr. In the intial experiments sulphuric acid was injected into the cultures and the carbon dioxide trapped in a vial of potassium hydroxide solution suspended above the culture. The resultant aqueous potassium carbonate was counted or alternatively the carbonate was precipitated as barium carbonate. Neither method proved satisfactory when standardized against a solution of sodium carbonate-[^{14}C] in the culture flask. Hence at the end of the experiment, after the addition of sulphuric acid, the gases were swept into a known volume of ethanolamine [17] which was then counted. This method when standardized against the carbon dioxide from sodium carbonate-[^{14}C] afforded a 98% recovery of radioactivity. A series of incubations were carried out and the gibberellins were recovered from the broth. TLC with radio scanning showed that gibberellins A_4/A_7 and gibberellic acid were the radioactive C_{19} gibberellins which were purified and counted. The results are given in Table 1 which shows the yield of $^{14}CO_2$ expected on the basis of the incorporation of *ent*-kaur-16-ene into the C_{19} gibberellins together with the observed yield. The filtered broths from two further incubations were also examined by dilution analysis for formaldehyde (as its dimedone derivative) and for formic acid (as its *p*-bromophenacyl derivative). In neither case were the derivatives radioactive.

The decomposition of gibberellic acid to allogibberic acid also yields carbon dioxide. The gibberellic acid formed from *ent*-kaur-16-ene biosynthesized from sodium acetate-[2^{14}C] will be labelled in the γ-lactone ring. Hence, in case this was the source of the labelled carbon

Table 1. Incubation of *ent*-kaur-16-ene-[^{14}C] with *Gibberella fujikuroi*

Expt.	Radio activity dpm × 10^{-4}						
	kaurene fed	gibberellic acid	gibberellins A_4/A_7	total C_{19} gibberellins	expected CO_2	found CO_2	% recovery
1	151.3	16.96	13.19	30.15	2.740	2.182	79.6
2	151.3	15.18	12.90	28.48	2.589	1.972	76.2



Scheme 1.

dioxide rather than C-20, the gibberellic-[^{14}C] acid was re-incubated with the fermentation for 24 hr. However no radioactive carbon dioxide was detected. Other potential sources such as the autoxidation of 19-aldehydes, can be excluded since the corresponding 19-norkaurenes are unknown as metabolites of *G. fujikuroi*. Hence the final fate of C-20 is as carbon dioxide.

A mechanism for this decarboxylation must account for a number of observations. Unlike sterol biosynthesis, mevalonoid hydrogen is retained [18, 19] at the centres, C-1, C-5, and C-9 adjacent to C-20 during the formation of the C_{19} gibberellins thus excluding unsaturated intermediates prior to the formation of the C_{19} gibberellins involving these centres. Furthermore the analogous steroidal C-19 is removed as formic acid rather than as carbon dioxide [12, 13]. Secondly both 19-oxygen atoms from gibberellin A_{12} (7-alcohol) are incorporated [20] into the γ-lactone ring of the C-19 gibberellins. Furthermore this γ-lactone ring lies on the same face of the molecule as the departing C-20 atom. Since the carbon atom eventually appears as carbon dioxide, we suggest that the decarboxylation might involve the decomposition of a C-20 per-acid by either a radical or ionic mechanism (Scheme 1).

EXPERIMENTAL

General fermentation procedure. Gibberella fujikuroi (ACC 917) was grown at 25° in shake culture (100 ml) on a medium comprising (per l.): glucose (80 g), NH_4NO_3 (0.48 g), KH_2PO_4 (5 g), $MgSO_4.7H_2O$ (1 g), trace elements soln (2 ml). The trace elements soln consisted of (per 100 ml) Fe $SO_4.7H_2O$ (0.1 g), $CuSO_4.5H_2O$ (0.015 g), $ZnSO_4.7H_2O$ (0.161 g), $MnSO_4.7H_2O$ (0.01 g), $(NH_4)_6MO_7O_{24}.4H_2O$ (0.01 g). After the fermentation, the mycelium was filtered, the broth was acidified to pH 1.5 (HCl), the metabolites were recovered by extraction (3 ×) with EtOAc (0.5 vol.). The mycelium was frozen (liq. N_2) and extracted (2 ×) with Me_2CO–EtOAc (1:1). *ent*-Kaur-16-ene was isolated from the broth and mycelial extracts by CC and TLC on Si gel in hexane. The gibberellins were purified by TLC developing first with hexane (tp remove *ent*-kaur-16-ene) and then with $CHCl_3$–EtOAc–AcOH (5:4:1). Samples were identified by comparison with authentic markers, eluted from the plates (EtOAc), crystallized and counted. Except where stated the *ent*-kaur-16-ene-[^{14}C] was material prepared biosynthetically from mevalonic acid-[2-^{14}C] AMO-1618 (2'-isopropyl-4'4 (trimethylammonium chloride)-5'methylphenylpiperidine-1-carboxylate) was obtained from Calbiochem.

Prepn of ent-*kaur*-16-*ene*-[^{14}C] *from sodium acetate*-[2-^{14}C].

Table 2. Incorporation of acetate-[2-^{14}C] into *ent*-kaur-16-ene by *Gibberella fujikuroi*

Age of culture when acetate-[2-^{14}C] fed (hr)	% Incorp. after 12 hr incubation	% Incorp. after 24 hr incubation
72	0.68	0.11
84	0.74	0.25
96	0.97	0.32
108	0.78	0.18

Sodium acetate-[2-^{14}C] (2.5 μCi) in sterile H_2O (0.1 ml) was fed to separate shake flasks of *Gibberella fujikuroi* at *ca* 72, 84, 96 and 108 hr from inoculation. These were harvested after a further 12 and 24 hr. The *ent*-kaurene was isolated and purified. The incorporations are listed in Table 2. Sodium acetate-[2-^{14}C] (2 mCi) in sterile H_2O (0.5 ml) was distributed between two 4-day-old cultures of *G. fujikuroi*. The cultures were harvested after a further 12 hr and the metabolites were purified [15] to afford *ent*-kaur-16-ene-[^{14}C] (22.7 μCi. 1.14% incorporation).

Effect of AMO-1618 on gibberellin biosynthesis. *ent*-Kaur-16-ene-[^{14}C] (3.56×10^6 dpm) at the time of inoculation. One *G. fujikuroi* grown in the presence of AMO-1618 (10^{-4} M). The culture was harvested after a further 3 days and the metabolites were isolated to afford gibberellic acid (2.84×10^6 dpm, 31.6% incorp.) and gibberellins A_4/A_7 (1.14×10^6 dpm, 12.6% incorp.).

Time-course study of the metabolism of ent-*kaur-16-ene.* Small (10 ml) cultures of *G. fujikuroi* were used throughout these experiments. (i) A group of 10 cultures were each fed *ent*-kaur-16-ene-[^{14}C] (3.56×10^6 dpm) at the time of inoculation. One culture was harvested daily. The *ent*-kaur-16-ene-[^{14}C] was recovered and the gibberellins A_3 and A_4/A_7 were isolated. The results are shown in Fig. 1. (ii) A group of ten of *G. fujikuroi* were inoculated. Each day one was fed with *ent*-kaur-16-ene-[^{14}C] (3.56×10^6 dpm) and this culture was then harvested 24 hr later. The *ent*-kaur-16-ene was recovered and the gibberellins A_3 and A_4/A_7 were purified. The results are shown in Fig. 2.

Incubations of ent-*kaur-16-ene*-[^{14}C] *prepared from sodium acetate*-[2-^{14}C] *with* G. fujikuroi. Four 4-day-old small (10 ml) cultures of *G. fujikuroi* containing AMO-1618 (10^{-4} M) were each fed *ent*-kaur-16-ene-[^{14}C] (1.51×10^6 dpm) which had been prepared biosynthetically from sodium acetate-[2-^{14}C] as described above. Two of the flasks were fitted with sterile taps to seal the flasks from the atmosphere for the period of the incubation. After 24 hr growth at the natural pH the broth was acidified with 5 N H_2SO_4 (5 ml) and the gases from these two cultures were swept with a stream of N_2 into traps containing ethanolamine (1.5 ml). The ethanolamine was diluted with MeOH (10 ml) and divided between two scintillation vials. A soln of 2,5-diphenyloxazole in xylene (9 g/l.) (5 ml) was added to each vial and the [^{14}C] counted. The counting efficiency was determined by the addition of hexadecane-[^{14}C] as an int. stand. The cultures were then filtered, and the gibberellins A_3 and A_4/A_7 were isolated, purified and the incorporation determined. The results are given in Table 1. The remaining two were filtered and the broth from each was divided into two portions. One portion from each culture was examined for the presence of radioactive formaldehyde [21], and the other for formic acid [22]. Formaldehyde (5 mg) was added to a portion of the broth, the pH adjusted to 4.5 and a soln of dimedone (80 ng) in 50% aq. EtOH (20 ml) was added. The soln was left at room temp. to crystallize. The dimedone derivative was filtered and recrystallized from EtOH. Expt. 1 gave formaldehyde dimethone (28.1 mg. 5.09 dpm/mg) and expt. 2 gave the dimethone (23.5 mg, 5.07 dpm/mg). Formic acid (46 mg) was added to a portion of the broth and the pH was adjusted to 9. A soln of *p*-bromophenacyl bromide (277 mg) in EtOH (20 ml) was added and the mixture was heated under reflux fof 30 min. The EtOH was removed *in vacuo*, and after the addition of H_2O (25 ml), the product was recovered in Et_2O and purified by PLC on Si gel in C_6H_6–EtOAc (19:1). Expt 1 gave 7.19 mg, 4.37 dpm/mg; expt. 2 gave 6.73 mg, 4.29 dpm/mg. In each case the derivatives were identified by comparison with authentic samples.

REFERENCES

1. Lew, F. T. and West, C. A. (1971) *Phytochemistry* **10**, 2065.
2. Hanson, J. R., Hawker, J. and White, A. F. (1972) *J. Chem. Soc. Perkin I* 1892.
3. Cross, B. E., Norton, K. and Stewart, J. C. (1968) *J. Chem. Soc. C* 1054.
4. Evans, R. and Hanson, J. R. (1975) *J. Chem. Soc. Perkin I* 663.
5. Bearder, J., MacMillan, J. and Phinney, B. O. (1975) *J. Chem. Soc. Perkin I* 721.
6. MacMillan, J. and Pryce, R. J. (1973) in *Phytochemistry* (Miller, L. P. ed.) Vol. 3, chap. 11. Van Nostrand–Reinhold, New York.
7. Olson, J. A., Lindberg, M. and Bloch, K. (1957) *J. Biol. Chem.* **226**, 941.
8. Gaylor, J. L. (1964) *J. Biol. Chem.* **239**, 756.
9. Swindell, A. C. and Gaylor, J. L. (1968) *J. Biol. Chem.* **243**, 5546.
10. Akhtar, M. and Skinner, S. J. M. (1968) *Biochem. J.* **109**, 318.
11. Skinner, S. J. M. and Akhtar, M. (1969) *Biochem. J.* **114**. 74.
12. Alexander, K., Akhtar, M. Boar, R. B., McGhie, J. F. and Barton, D. H. R. (1972) *J. Chem. Soc. Chem. Commun.* 383.
13. Arigoni, D., Battaglia, R. Akhtar, M. and Smith T. (1975) *J. Chem. Soc. Chem. Commun.* 185
14. Hanson, J. R. (1971) ***in Progress in the Chemistry of Organic*** *Natural Products* (Herz, W., Grisebach, H. and Kirby, G. W. eds) Vol. 29, p. 395. Springer-Verlag, Vienna.
15. Evans, R. and Hanson, J. R. (1972) *J. Chem. Soc. Perkin I* 2382.
16. Barnes, M. F., Light, E. N. and Lang, A. (1969) *Planta* **88**, 172.
17. Bosshart, R. E. and Young, R. K. (1972) *Anal. Chem.* **44**, 1117.
18. Hanson, J. R. and White, A. F. (1969) *J. Chem. Soc. C* 981.
19. Evans, R., Hanson, J. R. and White, A. F. (1970) *J. Chem. Soc. C* 2601.
20. Bearder, J. R., MacMillan, J and Phinney, B. O. (1976) *J. Chem. Soc. Chem. Commun.* 834.
21. LaDu, B. N., Gaudette, L., Trousof. H. and Brodie, B. B. (1955) *J. Biol. Chem.* **215**, 741.
22. Gabriel, O. (1965) *Anal Biochem.* **10**, 143.