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THE INHIBITION OF GIBBERELLIC ACID BIOSYNTHESIS BY *ENT*-KAURAN-16β,17-EPOXIDE

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

Abstract—*Ent*-kauran-16β,17-epoxide inhibits the biosynthesis of *ent*-kaur-16-ene from mevalonate and its conversion to gibberellic acid. It binds to a kaurene carrier protein.

INTRODUCTION

The inhibition of gibberellin plant hormone biosynthesis affords a direct approach to plant growth regulation. The well-known inhibitors of gibberellin biosynthesis, CCC, AMO-1618, phosphon-D, act on kaurene synthetase blocking the formation of the parent hydrocarbon, *ent*-kaur-16-ene (**1**) [1, 2]. More recent studies with, for example, ancymidol [3, 4] and 1-geranylimidazole [5], have led to the inhibition of the oxidative transformation of kaurene derivatives. The biosynthetic pathway leading to gibberellic acid (**2**) is now well-defined in *Gibberella fujikuroi*. Our objective has been to devise rational inhibitors of gibberellin plant hormone biosynthesis by the structural modification of known biosynthetic intermediates [6]. In this paper we report on the effect of *ent*-kauran-16β,17-epoxide (**3**) on gibberellin biosynthesis in *G. fujikuroi*.

RESULTS AND DISCUSSION

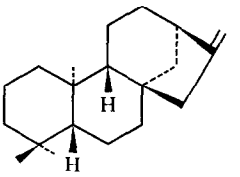
Ent-kaur-16-ene is an obligatory intermediate in gibberellin biosynthesis in the fungus *Gibberella fujikuroi* [7]. In studies with *Pisum sativum* [8], it has been reported to be attached to a carrier protein, whilst in the present work we have shown that it is associated with a protein fraction obtained from *G. fujikuroi*. Apart from steric factors associated with the overall structure, the double bond might provide an electron-rich binding group. We reasoned that the 16β,17-epoxide might bind more effectively by, for example, a hydrogen bond with a protein or perhaps irreversibly with a nucleophilic site (e.g. an -SH) and thus inhibit the formation and metabolism of *ent*-kaur-16-ene itself.

In order to define the concentrations at which inhibition might be obtained, we first determined by a GLC assay, the amount of *ent*-kaur-16-ene which was being produced by our cultures of *G. fujikuroi* at different stages of growth (Table 1). The optimum time (10–12 hr) for the incorporation of [2-^{14}C]-mevalonate into *ent*-kaur-16-ene was also established. *Ent*-kaur-16-ene was epoxidized with *m*-chloroperbenzoic acid to afford the known *ent*-kauran-16β,17-epoxide (**3**) [9, 10]. The epoxide was fed to 2-day-old cultures for a period of 12 hr at different concentrations (Table 2) and the *ent*-kaur-16-ene was purified. The epoxide clearly inhibited the formation of [^{14}C]-*ent*-kaur-16-ene from [2-^{14}C]-mevalonate. Furthermore, when *ent*-kauran-16β,17-epoxide and *ent*-[^{14}C]-kaur-16-ene (prepared biosynthetically from [2-^{14}C]-MVA) were fed to *G. fujikuroi*, the incorporation of the *ent*-[^{14}C]-kaur-16-ene into gibberellic acid, was also inhibited (Table 3).

Table 1. Production of *ent*-kaur-16-ene

Age of culture (days)	Amount of kaurene produced (mg/100 ml) (av. of 10 flask each)	
		s.d.
2	0.65	0.30
3	1.45	0.07
4	1.45	0.06
5	1.72	0.14
6	1.61	0.25
7	2.12	0.39

s.d. = standard deviation.



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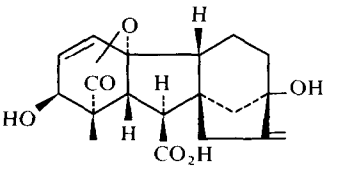

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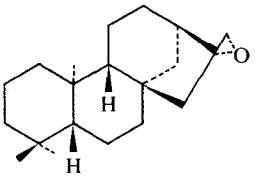

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Table 2. Effect of *ent*-kauran-16β,17-epoxide on the formation of ent-kaur-16-ene

Amount of epoxide added to each flask (mg)	Incorporation into kaurene (dpm)	% Incorporation of MVA into *ent*-kaur-16-ene (av. of 3 expts.)
Control	1.89×10^5	2.84 (±0.14)
1	1.18×10^5	1.79 (±0.12)
2	9.47×10^4	1.43 (±0.15)
4	5.08×10^4	0.77 (±0.16)
6	3.82×10^3	0.058 (±0.03)
8	—	0

A possible explanation for the apparent inhibition of both kaurene synthesis and metabolism is that the *ent*-kauran-16β,17-epoxide was binding to a carrier protein involved in the link between the soluble kaurene synthetase and the microsomal 19-oxidase. It could thus lead to the accumulation of kaurene on the synthetase and the consequent regulation of its biosynthesis. A cell-free preparation containing kaurene synthetase was prepared [11] from *G. fujikuroi* and incubated with [2-^{3}H]-MVA. The crude enzyme system was applied to a Sepharose 6B column which had been shown to separate protein from kaurene, the kaurane epoxide and from unchanged mevalonate. The protein fraction was split into two equal portions. One portion was denatured and the [^{3}H]-*ent*-kaur-16-ene was extracted. The other portion was incubated with *ent*-[^{14}C]-kauran-16β,17-epoxide for 3 hr and then repurified and counted. The amounts of ^{3}H and ^{14}C which were present indicated that the *ent*-[^{14}C]-kauran-16β,17-epoxide had displaced about 56% of the [^{3}H]-*ent*-kaur-16-ene. About 30% of the [^{14}C]-kauran-16β,17-epoxide was bound to the protein. The protein was denatured and both the [^{3}H]-*ent*-kaur-16-ene and the [^{14}C]-*ent*-kauran-16β,17-epoxide were separated and identified by co-chromatography with authentic samples. Thus the epoxide is reversibly binding to a protein which is involved in *ent*-kaur-16-ene metabolism.

When the *ent*-kauran-16β,17-epoxide was administered to young rice seedlings, it caused a slight (10–20%) retardation in growth (Table 4). When bio-assayed against a *G. fujikuroi* infection of rice seedlings, *ent*-kauran-16β,17-epoxide showed an inhibition of the characteristic 'bakanae' effect which is attributed to gibberellin production. In the light of these results we suggest that the use of appropriately substituted analogues of gibberellin biosynthetic intermediates can provide a rational approach to plant growth regulation.

EXPERIMENTAL

Gibberella fujikuroi ACC 917 was grown as described previously [12]. *Ent*-kauran-16β,17-epoxide was prepared from *ent*-kaur-16-ene with *m*-chloroperbenzoic acid in $CHCl_3$ at room temp. for 2 hr. It was purified by prep. TLC on Si gel in C_6H_6–hexane (1:9). The epoxide, identified by its ^{1}H NMR spectrum, had mp 114–116° (lit. [9, 10] 117°).

Ent-*kaur-16-ene production.* 12 Flasks (100 ml medium) of *G. fujikuroi* were inoculated and 2 flasks were harvested on day 2 and subsequently at 24 hr intervals. The mycelium was filtered, extracted with EtOAc and the extract concentrated to 1 ml. It was compared by GLC on SE-30 at 200° with a standard soln of kaurene in EtOAc. It was found by extraction of the broth that this contained less than 5% of the total kaurene. The results are given in Table 1.

Incubation with ent-*kauran-16β,17-epoxide.* The epoxide (Table 2) in EtOH (0.5 ml) was added to 2-day-old cultures of *G. fujikuroi* (3 flasks each concn) together with [2-^{14}C]-MVA (3.0 μCi per 3 flasks). After a further 12 hr, the mycelium was filtered and the broth acidified with dil HCl. Both were extracted with EtOAc and the *ent*-kaur-16-ene was purified by TLC on Si gel in C_6H_6–C_6H_{14} (1:9). The band of radioactivity which co-chromatographed with kaurene was eluted from the plate using EtOAc. The extract was mixed with kaurene (20 mg) and purified by CC on Si gel. Elution with petrol gave kaurene which was

Table 3. Effect of *ent*-kauran-16β,17-epoxide on the incorporation of *ent*-kaur-16-ene into gibberellic acid (GA) (average of 4 runs)

Age of culture after feed (days)	Control [^{14}C]-kaurene only		+2 mg epoxide per flask		+4 mg epoxide per flask	
	dpm × 10^{-5} in GA	% incorp.	dpm × 10^{-5} in GA	% incorp.	dpm × 10^{-5} in GA	% incorp.
2	3.98	4.5 (±0.60)	1.79	2.03 (±0.14)	0.763	0.86 (±0.25)
3	3.90	4.4 (±0.80)	1.66	1.88 (±0.38)	1.03	1.16 (±0.25)
4	4.70	5.34 (±0.27)	2.01	2.28 (±0.18)	1.32	1.50 (±0.22)
5	5.01	5.69 (±0.46)	2.36	2.68 (±0.16)	1.52	1.73 (±0.15)

Table 4. Effect of *ent*-kauran-16β,17-epoxide on the growth of rice seedlings

	Average height (cm)				No. of seedlings in sample
	5 days	s.d.	12 days	s.d.	
Control	3.69	1.02	8.19	2.61	121
+ *ent*-kauran-16β,17-epoxide	2.91	0.87	6.89	2.30	130
+ *Gibberella fujikuroi*	8.19	1.43	12.13	3.06	137
+ *Gibberella fujikuroi* and *ent*-kauran-16β,17-epoxide	5.66	1.07	10.33	2.84	105

recrystallized to constant radioactivity (mp 50–52°). On some occasions the product was again chromatographed using TLC and scanned for radioactivity. The results are given in Table 2.

The incorporation of ent-*kaur*-16-*ene into gibberellic acid.* 16 Flasks (100 ml medium) were inoculated with *G. fujikuroi*. After 1 day's growth, 8 flasks were fed with [^{14}C]-*ent*-kaur-16-ene (2 μ Ci per flask) in EtOH (0.1 ml) whilst the other 8 flasks were fed with [^{14}C]-*ent*-kaur-16-ene (2 μ Ci per flask) and *ent*-kauran-16β,17-epoxide (2 mg) in EtOH (0.25 ml) per flask). After a further 2, 3, 4 and 5 day's growth, 2 cultures of each were harvested and the gibberellic acid was recovered in EtOAc. The extract was separated by TLC on Si gel in $CHCl_3$–EtOAc–HOAc (5:4:1). The band of radioactivity which co-chromatographed with gibberellic acid was eluted from the plate with EtOAc and methylated with CH_2N_2. The ester was mixed with methyl gibberellate (10 mg), chromatographed on Si gel in $CHCl_3$–C_6H_6–$MeCO_2H$ (7:2:1) and crystallized to constant sp. act. from EtOAc–petrol. The experiment was repeated using 4 mg *ent*-kauran-16β,17-epoxide per flask. The results (the average of 4 runs) are given in Table 3.

Protein binding experiments. A cell-free system was prepared from 6 flasks (100 ml medium) of a 3-day-old culture of *G. fujikuroi* as described previously [11]. It was incubated with [2-^{3}H]-MVA (1 μ Ci) for 3 hr at 30°. The soln (3 ml) was applied to a Sepharose 6B column (21 × 2.2 cm) and eluted with a 0.1 M phosphate buffer. The protein fractions (determined by 250 nm absorption) were eluted after the void volume. These fractions (3 × 3 ml) were divided into two portions. One was denatured using liquid N_2 and Me_2CO and extracted with EtOAc. The extract was purified by TLC on Si gel in C_6H_6–C_6H_{14} (1:9) to afford [^{3}H]-*ent*-kaur-16-ene. The band which co-chromatographed with authentic kaurene was eluted from the plate with EtOAc, diluted with kaurene (10 mg) and crystallized to constant sp. act. (5.6 × 10^4 dpm, s.d. 8.6 × 10^3; 2.5 ± 0.37% incorp. of ^{3}H; average of 4 runs). The other portion was incubated with *ent*-[^{14}C]-kauran-16β,17-epoxide (2.5 mg; 60000 dpm) in EtOH (0.2 ml) and Tween 80 (1 drop) for 3 hr at 30°. The protein fraction was repurified on a Sepharose 6B column (21 × 2.2 cm) and then denatured using liquid N_2 and Me_2CO and extracted with EtOAc. The extract was separated by TLC on Si gel in C_6H_6–C_6H_{14} (1:9). Two bands were obtained. The less polar co-chromatographed with kaurene, the more polar with the epoxide. Both bands were eluted from the plate with EtOAc, each was mixed with 10 mg authentic material and crystallized to constant radioactivity. The [^{3}H]-kaurene had 1.79 × 10^4 dpm (s.d. 1.34 × 10^3 dpm; 44% of the original radioactivity). The [^{14}C]-kaurane epoxide had 1.8 × 10^4 dpm (s.d. 1.32 × 10^3 dpm; 29% incorp.). The other fractions from the Sepharose column were examined for radioactivity and purified by TLC to afford [^{3}H]-kaurene (1.53 × 10^4 dpm, s.d. 1.81 × 10^3 dpm) and [^{14}C]-kaurane epoxide (3.91 × 10^4 dpm, s.d. 2.68 × 10^3 dpm).

Bioassay experiments. Rice seedlings were germinated in conical flasks (500 ml) on an agar medium (200 ml) containing $CaSO_4$ (0.25 g), $Ca_3(PO_4)_2$ (0.25 g), $MgSO_4$ (0.25 g), NaCl (0.08 g), KNO_3 (0.7 g), $FeCl_3$ (0.005 g) and chloramphenicol (50 mg). The seeds were surface-sterilized with 10% NaClO and then rinsed with sterile water. Seeds (*ca* 30) were placed in each flask. 4 Flasks were retained as a control, 4 flasks were treated with a 1-week-old culture of *G. fujikuroi* (2 ml each flask), 4 flasks were treated with *ent*-kauran-16β,17-epoxide (4 mg) in the minimum of EtOH, Tween 80 and water (2 ml) and 4 flasks were treated with both the *G. fujikuroi* and the *ent*-kauran-16β,17-epoxide. The average height of each group of the seedlings on day 5 and 12 is given in Table 4.

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REFERENCES

1. Cross, B. E. (1968) in *Progress in Phytochemistry*, Vol. 1, p. 195. Wiley, London.
2. Corcoran, M. R. (1975) in *Gibberellins and Plant Growth*, Chap. 12, p. 289. Wiley, New Dehli.
3. Coolbaugh, R. C. and Hamilton, R. (1976) *Plant Physiol.* **57**, 245.
4. Shive, J. B. and Sisler, H. D. (1976) *Plant Physiol.* **57**, 640.
5. Wada, K. (1978) *Agric. Biol. Chem. (Jpn.)* **42**, 2411.
6. Corey, E. J. and Volante, R. P. (1976) *J. Am. Chem. Soc.* **98**, 1291.
7. Cross, B. E., Galt, R. H. B. and Hanson, J. R. (1964) *J. Chem. Soc.* 295.
8. Moore, T. C., Barlow, S. A. and Coolbaugh, R. C. (1972) *Phytochemistry* **11**, 3225.
9. Briggs, L. H., Cambie, R. C. and Rutledge, P. S. (1963) *J. Chem. Soc.* 5374.
10. Hanson, J. R. (1967) *Tetrahedron* **23**, 801.
11. Evans, R. and Hanson, J. R. (1972) *J. Chem. Soc. Perkin Trans.* **1**, 2382.
12. Dockerill, B. and Hanson, J. R. (1978) *Phytochemistry* **17**, 701.