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THE MICROBIOLOGICAL TRANSFORMATION OF SOME *ENT*-KAUR-6, 16-DIENES BY *GIBBERELLA FUJIKUROI*

BRAULIO M. FRAGA, ANTONIO G. GONZALEZ, PEDRO GONZALEZ, JAMES R. HANSON* and MELCHOR G. HERNANDEZ

Instituto de Productos Naturales del CSIC, La Laguna, Tenerife, Spain; *School of Molecular Sciences, University of Sussex, Brighton, Sussex, U.K.

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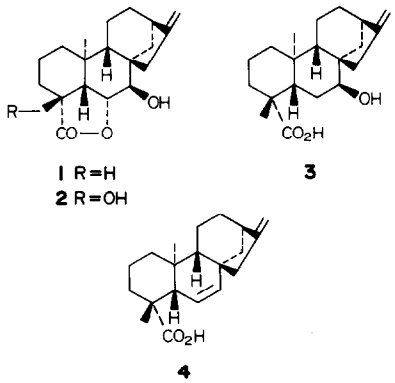
Key Word Index—*Gibberella fujikuroi*; *ent*-kaur-6, 16-dienes; *ent*-6α, 7α-epoxy-3β, 18-dihydroxykaur-16-ene; kaurenolide biosynthesis.

Abstract—The preparation of *ent*-kaur-6, 16-diene and *ent*-18-hydroxykaur-6, 16-diene from epicandicandiol and of *ent*-3β, 18-dihydroxykaur-6, 16-diene from foliol is described. On incubation with *Gibberella fujikuroi ent*-kaur-6, 16-diene gave 7-hydroxykaurenolide and 18-hydroxykaur-6, 16-diene afforded 7, 18-dihydroxykaurenolide, whilst *ent*-6α,7α-epoxy-3β,18-dihydroxykaur-16-ene was obtained from *ent*-3β,18-dihydroxykaur-6,16-diene. The possible biosynthetic significance of the 6, 7-epoxidation in kaurenolide formation is noted.

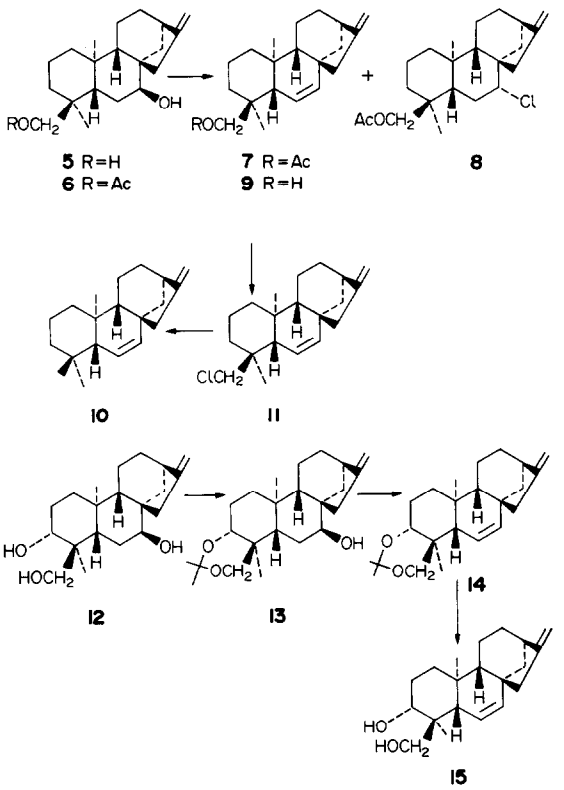
INTRODUCTION

The fungus, *Gibberella fujikuroi*, is capable of transforming various artificial substrates along sections of its diterpenoid metabolic pathways. Some interesting biosynthetic results have accrued from these investigations. The metabolites of *Gibberella fujikuroi* include both the gibberellins and the kaurenolides of which 7-hydroxykaurenolide (**1**) [1] and 7,18-dihydroxykaurenolide (**2**) [2] are the major members. The stage at which the gibberellin and kaurenolide pathways diverge has recently been the subject of some interest [3, 4]. The kaurenolide lactones possess a *trans*-diaxial diol on ring B. In earlier biosynthetic studies [5] it had been shown that *ent*-7α-hydroxykaurenoic acid (**3**) was converted into the lactones. In the light of more recent results it was clear that the incorporation (0.03 and 0.44%, respectively) was low compared to the incorporation into gibberellic acid (32.3%), despite the fact that only one or two biosynthetic steps were involved in the formation of the kaurenolides. Recently *ent*-kaur-6, 16-dien-19-oic acid (**4**) rather than ent-7α-hydroxykaurenoic acid (**3**) has been shown to be an efficient precursor of 7-hydroxykaurenolide (**1**) in *Cucurbita maxima* [3] and in a mutant of *Gibberella fujikuroi* [4]. It is probable that our earlier result may have the status of a microbiological conversion. In order to account for the formation of the kaurenolides from the 6-enes, it was suggested [4] that a 6,7-epoxide was formed first and that this underwent a *trans*-diaxial ring opening with participation of the 19-carboxyl group to generate the lactone ring. However, although both the 6α,7α- and 6β,7β-epoxides can be prepared [6] chemically in the presence of a 19-methyl ester, it was not possible to detect a 6,7-epoxide *in vivo*, presumably owing to its rapid hydrolysis in the presence of a 19-carboxylic acid. Recently we have shown [7] that a 3α-hydroxyl group inhibits the microbiological oxidation of C-19 by *Gibberella fujikuroi*. We reasoned that a comparison of the metabolism of *ent*-kaur-6, 16-dienes with *ent*-kaur-16-enes in the presence and absence of this 3α-blocking group, might shed some further light on this stage. Part of this work has been the subject of a preliminary communication [8].



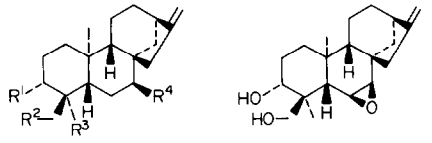
RESULTS AND DISCUSSION

The substrates were prepared as shown in Scheme 1. Epicandicandiol (*ent*-7α, 18-dihydroxykaur-16-ene) (**5**) was converted to its 18-monoacetate (**6**) [9]. The 7-hydroxyl group was then eliminated by treatment of the toluene-*p*-sulphonate with collidine or by reaction with triphenylphosphine–carbon tetrachloride–pyridine to afford *ent*-18-acetoxykaur-6, 16-diene (**7**). The multiplicity of the olefinic proton resonances in the ^{1}H NMR spectrum (δ 5.40, H-6, *q*, $J_{5-6} = 3$ Hz, $J_{6-7} = 11$ Hz; δ 5.57, H-7, *d*, $J_{6-7} = 11$ Hz) established that no rearrangement had taken place. The 7α-chloro compound (δ 4.00 H-7, *q*, $J = 7$ Hz) (**8**) accompanied the alkene in the triphenylphosphine reaction. Hydrolysis of the 18-acetoxyl group with methanolic potassium hydroxide afforded *ent*-18-hydroxykaur-6, 16-diene (**9**) (δ 3.10 and 3.36, H-18, *d*, $J = 11$ Hz). The hydrocarbon, *ent*-kaur-6, 16-diene (**10**) was prepared from this by reaction with triphenylphosphine–carbon tetrachloride–pyridine and reduction of the resultant 18-chloro-compound (**11**) with tri-*n*-butyl tin hydride.



Scheme 1. The preparation of kaur-6, 16-dienes.

ent-3β, 18-Dihydroxykaur-6, 16-diene was prepared from foliol (*ent*-3β,7α,18-trihydroxykaur-16-ene) (**12**). The 3- and 18-hydroxyl groups were protected as the acetonide (**13**) [7] whilst the 7-hydroxyl group was eliminated by treatment with triphenylphosphine–carbon tetrachloride–pyridine to afford the 6-ene (**14**). Hydrolysis of the acetonide with aqueous ethanolic hydrochloric acid gave *ent*-3β,18-dihydroxykaur-6,16-diene (**15**).



16 $R^1=R^2=H$, $R^3=Me$, $R^4=OH$
17 $R^1=H$, $R^2=OH$, $R^3=Me$, $R^4=H$
18 $R^1=H$, $R^2=R^4=OH$, $R^3=Me$
19 $R^1=H$, $R^2=R^4=OH$, $R^3=CH_2OH$
20 $R^1=H$, $R^2=R^4=OH$, $R^3=CO_2H$
21 $R^1=OH$, $R^2=R^4=H$, $R^3=Me$
22 $R^1=R^2=OH$, $R^3=Me$, $R^4=H$
23 $R^1=R^2=R^4=OH$, $R^3=Me$

24

The role of the 6-ene in directing the metabolism of these substrates was best assessed by comparison of their fate with that of their saturated and 7-hydroxy analogues. The detection of the metabolites of post-kaurene artificial substrates is facilitated by growing *G. fujikuroi* in the presence of AMO-1618 which blocks the formation of endogenous *ent*-kaur-16-ene [10, 11]. Previously we have shown [12] that incubation of *ent*-7α-hydroxykaur-16-ene (**16**) with *G. fujikuroi* afforded products which were characteristic of the normal metabolism of *ent*-7α-hydroxykaurenoic acid (**3**). However, we were unable to detect any kaurenolides. On the other hand *ent*-18-hydroxykaur-16-ene (**17**) gave 7,18-dihydroxykaurenolide (**2**). When the hydroxylation pattern was combined in *ent*-7α,18-dihydroxykaur-16-ene (**18**) there was no evidence [13], by radio-isotope dilution analysis, for the formation of the kaurenolide (**2**). Thus, although minor pathways may exist, an *ent*-7α-hydroxy group was incompatible with efficient microbiological transformation into the kaurenolide branch of the diterpenoid pathways.

Whereas *ent*-kaur-16-ene is an efficient precursor of both the kaurenolides and the gibberellins [14], incubation of *ent*-kaur-6, 16-diene (**10**) with *G. fujikuroi* gave 7-hydroxykaurenolide (**1**) and 7,18-dihydroxykaurenolide (**2**) but no detectable gibberellin metabolites. Similarly, *ent*-18-hydroxykaur-6, 16-diene (**9**) gave 7,18-dihydroxykaurenolide (**2**) and, surprisingly, some *ent*-3β,18-dihydroxykaur-6,16-diene (**15**). The kaurenolides were identified by their NMR and mass spectra. Thus, in contrast to the effect of an *ent*-7α-hydroxy group, the presence of a 6-ene appears to direct microbiological transformation into the kaurenolide pathway paralleling the biosynthetic results [3, 4].

The major metabolites of *ent*-7α, 18-dihydroxykaur-16-ene (**18**) were the 19-alcohol (**19**) and the 19-acid (**20**) [13]. On the other hand *ent*-3β-hydroxykaur-16-ene (**21**) was not metabolized [7], whilst *ent*-3β, 18-dihydroxykaur-16-ene gave *ent*-3β,7α, 18-trihydroxykaur-16-ene (**23**). Thus, hydroxylation at C-19 by *G. fujikuroi* is being inhibited by an *ent*-3β-hydroxyl group. Incubation of *ent*-3β, 18-dihydroxykaur-6, 16 diene (**15**) with *G. fujikuroi* gave a good yield of *ent*-6α, 7α-epoxy-3β, 18-dihydroxykaur-16-ene (**24**). The structure and stereochemistry of the epoxide followed from the ^{1}H NMR spectrum. The olefinic resonance of the starting material (δ 5.45) was replaced by epoxide signals at δ 2.90 (H-7, *d*, J = 4 Hz) and 3.04 (H-6, *q*, J = 2.5 and 4 Hz). The magnitude (2.5 Hz) of the H-5, H-6 coupling is consistent with a β-epoxide [6]. The structure of this metabolite, and in particular the stereochemistry of the epoxide ring, was confirmed by X-ray analysis [8].

Microbiological epoxidation of an alkene is sometimes equivalent to the hydroxylation of the corresponding alkane [15]. The ring contraction step in gibberellin biosynthesis involves the loss of the hydroxyl hydrogen and the C-6β-hydrogen atom, whereas kaurenolide formation apparently involves the loss of the 6α- and 7β-hydrogen atoms [16]. The divergence in the oxidative modification of ring B in kaurenolide–gibberellin biosynthesis may depend upon the order in which dehydrogenation and hydroxylation (epoxidation) occur.

EXPERIMENTAL

General exptal details have been described previously [5, 13].

ent-18-*Acetoxykaur*-6, 16-*diene* (**7**). (a) Epicandicandiol 18-monoacetate (**6**) (1.0 g) [9] and toluene-*p*-sulphonyl chloride (2.5 g) in dry C_5H_5N (10 ml) were left at room temp. for 3 days. The soln was diluted with H_2O and the product recovered in EtOAc. The solvent was evaporated and the residue refluxed with collidine (8 ml) for 1 hr. The soln was cooled, poured into H_2O and the product recovered in EtOAc and chromatographed on Si gel. Elution with petrol–EtOAc (9:1) gave *ent*-18-acetoxykaur-6,

16-diene (**7**) (900 mg), mp 92–94°, δ ($CDCl_3$): 0.97 and 0.87 (each 3H, *s*, H-19 and H-20), 2.03 (3H, *s*, OAc), 3.79 (2H, *s*, H-18), 4.82 (2H, *br s*, H-17), 5.40 (1H, *q*, J = 3 and 11 Hz, H-6), 5.57 (*d*, J = 11 Hz., H-7); MS: 328 $[M]^+$, 268 $[M-60]^+$, 250, 239, 225, 211, 199, 197, 183, 169, 143.

(b) Epicandicandiol 18-monoacetate (**6**) (1.2 g) in dry C_5H_5N (23 ml) and CCl_4 (90 ml) containing triphenylphosphine (2.5 g) was heated under reflux for 75 min. The solvent was evaporated and the products chromatographed on Si gel. Elution with petrol–EtOAc (49:1) afforded *ent*-18-acetoxykaur-6, 16-diene (**7**) (940 mg) followed by *ent*-18-acetoxy-7α-chlorokaur-16-ene (**8**) (40 mg) as a gum, δ ($CDCl_3$): 0.83 and 1.08 (each 3H, *s*, H-19 and H-20), 2.05 (3H, *s*, OAc), 3.67 and 3.83 (each 1H, *d*, J = 11 Hz, H-18), 4.00 (1H, *q*, J = 7 Hz, H-7), 4.80 (2H, *br s*, H-17).

ent-18-*Hydroxykaur*-6, 16-*diene* (**9**). *ent*-18-Acetoxykaur-6, 16-diene (**7**) (472 mg) was treated with 5% methanolic KOH (20 ml) at room temp. overnight. The soln was neutralized and the product recovered in EtOAc to afford *ent*-18-hydroxykaur-6, 16-diene (**9**) (415 mg) as a gum. (Found: M^+ 286.2266. $C_{20}H_{30}O$ requires: 286.2297.) δ ($CDCl_3$): 0.77 and 0.97 (each 3H, *s*, H-19 and H-20), 3.10 and 3.36 (each 1H, *d*, J = 11 Hz, H-18), 4.77 (2H, *br s* H-17), 5.37 (1H, *q*, J = 3 and 9 Hz, H-6), 5.55 (2H, *d*, J = 9 Hz, H-7); MS: 286, 255, 225, 199, 155, 143.

ent-18-*Chlorokaur*-6, 16-*diene* (**11**). The above alcohol (**9**) (800 mg) in dry C_5H_5N (10 ml) and CCl_4 (70 ml) was treated with triphenylphosphine (1 g) under reflux for 3 hr. The solvent was evaporated and the residue chromatographed on Si gel. Elution with petrol–EtOAc (9:1) afforded *ent*-18-chlorokaur-6, 16-diene (**11**) (650 mg) as a gum. (Found: M^+ 304.1915, $C_{20}H_{29}Cl$ requires: 304.1957.) δ ($CDCl_3$): 0.94 and 0.97 (each 3H, *s*, H-19 and H-20) 3.36 (2H, *s*, H-18), 4.84 (2H, *br s*, H-17), 5.47 (2H, *s*, H-6 and H-7); MS: 304, 289, 263, 261, 255, 233, 221, 213, 199, 185, 173.

ent-*Kaur*-6, 16-*diene* (**10**). *ent*-18-Chlorokaur-6, 16-diene (**11**) in C_6H_6 (30 ml) was treated with tri-*n*-butyltin hydride (1 ml) and azobisisobutryonitrile (5 mg) under reflux for 7 hr. The soln was diluted with Et_2O and H_2O satd with KF [17] and left for 15 min. After filtration the soln was dried and evaporated to afford *ent*-kaur-6, 16-diene (**10**) (400 mg), mp 50–51°. (Found: 270.2339. $C_{20}H_{30}$ requires: 270. 2348.) δ ($CDCl_3$): 0.85, 1.00 and 1.05 (each 3H, *s*, H-18–H-20), 4.83 (2H, *br s* H-17), 5.41 (1H, *q*, J = 3 and 9 Hz, H-6), 5.63 (1H, *d*, J = 9 Hz, H-7); MS: 270, 255, 227, 199, 185, 173, 157, 143, 131, 129.

ent-3β, 18-*Acetonidokaur*-6, 16-*diene* (**14**). Foliol acetonide (**13**) (900 mg) [7] was treated as above with triphenylphosphine (2.2 g), CCl_4 (80 ml) and C_5H_5N (17 ml) for 2.5 hr to afford, after chromatography, *ent*-3β, 18-acetonidokaur-6, 16-diene (**14**) (800 mg), mp 184–186°. (Found: 342.2507. $C_{22}H_{34}O_2$ requires: 342.2558.) δ ($CDCl_3$): 1.01 and 1.09 (each 3H, *s*, H-19 and H-20), 1.43 (6H, *s*, acetonide), 3.43 and 3.61 (each 1H, *d*, J = 11 Hz, H-18), 3.49 (1H, *m*, H-3), 4.85 (2H, *br s*, H-17), 5.34 (1H, *q*, J = 3 and 11 Hz, H-6), 5.48 (1H, *d*, J = 11 Hz); MS: 342, 327, 284, 267, 239, 227, 211, 199.

ent-3β, 18-*Dihydroxykaur*-6, 16-*diene* (**15**). The above acetonide (**14**) (700 mg) in EtOH (50 ml) was treated with 10% aq. ethanolic HCl (1 ml) for 2 hr at room temp. The soln was poured into H_2O and the product recovered in EtOAc to afford *ent*-3β, 18-dihydroxykaur-6, 16-diene (**15**) (560 mg), mp 140–142°. (Found: 302.2253. $C_{20}H_{30}O_2$ requires: 302.2245.) δ ($CDCl_3$): 0.90 and 0.99 (each 3H, *s*, H-19 and H-20), 3.40 and 3.72 (each 1H, *d*, J = 11 Hz, H-18), 4.80 (2H, *br s*, H-17), 5.49 (2H, *s*, H-6 and H-7); MS: 302, 284, 269, 266, 251, 241, 223, 211, 209, 199.

Incubation expts. (a) *Gibberella fujikuroi*, inhibited with 5×10^{-5} M AMO 1618, was grown in shake culture at 25° for 1 day in 80 conical flasks (250 ml) each containing sterile medium (50 ml) [5]. *ent*-3β, 18-Dihydroxykaur-6, 16-diene (390 mg) in EtOH (20 ml) was distributed equally between 75 flasks and the remaining five flasks were retained as a control. The incubation was allowed to continue for a further 3.5 days. The broth was filtered, acidified with dilute HCl and extracted with EtOAc. The extract was separated into acidic and neutral fractions with aq. $NaHCO_3$. TLC revealed that the major metabolite was in the neutral fraction which was chromatographed on Si gel. Elution with EtOAc gave *ent*-6α, 7α-epoxy-3β, 18-dihydroxykaur-16-ene (**24**) (170 mg), mp 162–164°. (Found: C, 74.6; H, 8.4, M^+ 318.2183. $C_{20}H_{30}O_3$ requires: 75.4; H, 9.5% M^+ 318.2195.) δ ($CDCl_3$): 1.04 and 1.07 (each 3H, *s*, H-19 and H-20), 2.90 (1H, *d*, J = 4 Hz, H-7), 3.04 (1H, *q*, J = 2.5 and 4 Hz, H-6), 3.60, 3.82 (each 1H, *d*, J = 11 Hz, H-18), 3.59 (1H, *t*, H-3), 4.92 (2H, *br s*, H-17); MS: 318, 303, 300, 287, 270, 255, 241, 227, 119.

(b) *ent*-18-Hydroxykaur-6, 16-diene (253 mg) in EtOH (20 ml) was distributed equally between 75 flasks and incubated as above. The products were recovered in EtOAc. The neutral fraction was chromatographed on Si gel. Elution with EtOAc–petrol (7:13) gave 7, 18-dihydroxykaurenolide (**2**) (15 mg) identified by comparison (TLC, ^1H NMR and MS with authentic material. Further elution with EtOAc gave the epoxide (**24**) (19 mg) identical with the material obtained above. The feeding of *ent*-18-hydroxykaur-6, 16-diene was repeated twice with similar results. The mycelium from the three incubations was extracted with $CHCl_3$ in a Soxhlet thimble. The extract was chromatographed on Si gel to afford *ent*-3β, 18-dihydroxykaur-6, 16-diene (**15**) (15 mg), mp 135–138° (Found: 302.2235. $C_{20}H_{30}O_2$ requires: 302.2246.) identical (TLC, MS and NMR) to an authentic sample.

(c) *ent*-Kaur-6, 16-diene (**10**) (303 mg) in EtOH (20 ml) was distributed equally between 75 flasks and incubated as above. The broth was extracted with EtOAc and the metabolites separated into acidic and neutral fractions. The neutral fraction was chromatographed on Si gel. Elution with EtOAc–petrol (3:17) gave 7β-hydroxykaurenolide (**1**) (14 mg) identified (TLC, NMR, MS) by comparison with authentic material. Further elution with EtOAc–petrol (3:7) gave 7, 18-dihydroxykaurenolide (**2**) identical to that obtained from *ent*-18-hydroxykaur-6, 16-diene.

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