Studies in Terpenoid Biosynthesis. Part 23.[1] Relationships between the Kaurenolides and the Seco-ring B Metabolites of *Gibberella fujikuroi*

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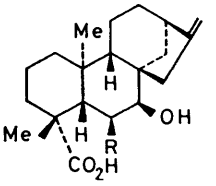
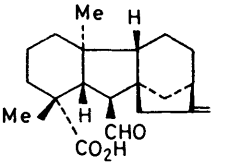
The hydroxylation at C-11 and C-18, oxidation of C-18, and cleavage of ring B of some kaurenolides by *Gibberella fujikuroi* is described.

DITERPENOID metabolism in the fungus *Gibberella fujikuroi* involves a series of divergent pathways in which *ent*-7α-hydroxykaur-16-en-19-oic acid (1) forms one important branching point.[2] Abstraction of the 6β-hydrogen atom [3] is accompanied by ring contraction to form gibberellin A_{12} aldehyde (2), whilst loss of the 6α-hydrogen atom affords the kaurenolide lactones, *e.g.* 7-hydroxykaurenolide (3). In previous papers [4] we and others [5] have examined the relationship between the gibberellins in the fungus. In this paper we are concerned with the relationship between the kaurenolides [6] and the unusual aldehyde anhydride, fujenal (4).[7]

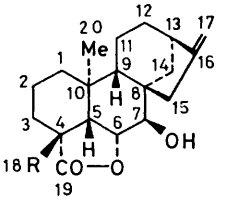
7,18-Dihydroxykaurenolide (5) [8] is the major kaurenolide metabolite of *G. fujikuroi* strain ACC 917. It is accompanied by smaller amounts of 7-hydroxykaurenolide (3). Other kaurenolides such as 3β,7β-dihydroxykaurenolide,[9,10] 7β,11α-dihydroxykaurenolide,[10] 7β,13-dihydroxykaurenolide,[11a] and 4β,7β-dihydroxy-18-norkaurenolide [11b] have been detected in different strains of the fungus although kaurenolide itself has not been



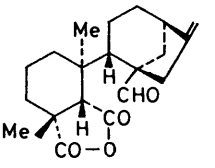
(1) R = H
(14) R = OH



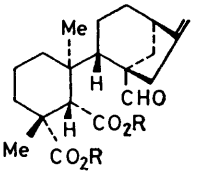
(2)



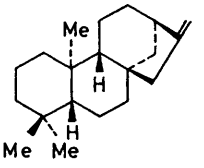
(3) R = Me
(5) R = CH_2OH
(7) R = CO_2Me
(8) R = H
(9) R = OH



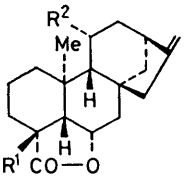
(4a)



(4b) R = H
(4c) R = Me



(6)



(10) R^1 = CH_2OH, R^2 = H
(11) R^1 = H, R^2 = OH
(12) R^1 = CO_2Me, R^2 = OH
(13) R^1 = CO_2H, R^2 = OH

recorded as a natural metabolite of the fungus. The metabolite, fujenal, is obtained either as the aldehyde anhydride (4a) when it occurs in the neutral fractions or as the aldehyde dicarboxylic acid (4b) when it appears in the acidic fractions.[7] Thus heating fujenal in water for 15 min affords the diacid. The aldehyde dicarboxylic acid (4b) may be trapped as the dimethyl ester (4c) through methylation with diazomethane; otherwise on subsequent silica chromatography the aldehyde anhydride form of fujenal is obtained.

ent-Kaur-16-ene (6) has been shown [12] to be an efficient precursor of 7,18-dihydroxykaurenolide (5). The plant-growth regulator, AMO-1618, inhibits [13] the formation of *ent*-kaur-16-ene in *G. fujikuroi* and hence endogenous kauranoid metabolites are not formed, facilitating the detection of metabolites from added substrates. However, the plant growth regulator does not appear to modify the subsequent metabolism of *ent*-kaur-16-ene by *G. fujikuroi*.[14] Although caution has to be exercised in the interpretation of metabolic results based on the use of inhibitors or enzymatically deficient mutants, because of the possibility of induced biosyntheses, nevertheless the method is simply obviating the need for labelling potential precursors. Hence unlabelled substrates were fed to the fungus in the presence of AMO 1618. The metabolites were isolated and compared (t.l.c.) to parallel control fermentations. New metabolites were then purified by column chromatography.

RESULTS AND DISCUSSION

7-Hydroxykaurenolide (3) is the first kaurenolide to be formed from *ent*-7α-hydroxykaur-16-en-19-oic acid (1).[2] It was fed to a fermentation of *G. fujikuroi* in the presence of AMO 1618. The major products which were isolated and identified (i.r. and n.m.r.) were fujenal (4) and 7,18-dihydroxykaurenolide (5) (*cf.* ref. 15). To avoid separation problems, the fujenal was isolated as the aldehyde dimethyl ester (4c) *via* the acidic fraction. The further metabolism of 7,18-dihydroxykaurenolide (5) was then examined. The major product was an unstable acidic compound. This was therefore purified as its methyl ester, obtained by methylating the total acidic extract with diazomethane. The n.m.r. spectrum of this compound lacked the signals associated with the C-18 hydroxymethyl group, containing instead a new methoxy group, and hence it was assigned the structure (7). This was confirmed by its partial synthesis from 7,18-dihydroxykaurenolide by the selective chemical oxidation of C-18.[16] No compounds of an 18-hydroxy-fujenal type were detected in the fermentation.

Decarboxylation of the 18-acid corresponding to (7) readily afforded 7-hydroxy-18-norkaurenolide (8) and hence this was examined as a substrate. The major metabolite, $C_{19}H_{26}O_4$, contained an additional tertiary hydroxyl group (no additional C*H*–O signal in the ^1H n.m.r.; new SFORD singlet *C*–O resonance in the ^{13}C n.m.r.). The location of this hydroxy group was established by ^{13}C n.m.r. (see Table). In particular, when compared to (7), the resonance assigned to C-4 had moved downfield to 73.7 p.p.m. whilst the signals associated with C-3 and C-5 showed downfield shifts consonant with the introduction of an adjacent hydroxy group. Hence the metabolite was 4β,7β-dihydroxy-18-norkaurenolide (9). It had physical constants in agreement with those in the literature.[11]

^{13}C N.m.r. resonances of some kaurenolides (solvent $CDCl_3$, p p.m. from $SiMe_4$)

Carbon	(5)	(7)	(8)	(9) *	(10)	(12)
1	36.9	36.3	37.3	38.2	39.8	38.8
2	17.4	17.0	16.8	18.6	17.0	17.1
3	23.5	24.3	19.1	30.3	22.9	24.8
4	45.9	54.0	37.9	73.7	51.9	53.9
5	48.5	48.3	44.8	52.8	38.2	48.5
6	85.7	84.9	85.0	85.7	53.8	77.0
7	70.9	71.3	71.7	70.9	36.6	36.8
8	49.1	45.2	45.5	46.3	35.9	40.3
9	55.8	55.2	54.7	55.0	48.1	57.0
10	34.3	33.7	33.7	34.8	34.5	35.6
11	17.4	17.0	17.7	17.2	17.0	65.0
12	32.9	32.5	32.6	32.9	32.9	45.6
13	38.2	37.5	37.6	37.7	36.6	38.2
14	34.3	33.9	34.2	34.9	38.5	34.9
15	42.5	41.9	42.2	42.8	48.4	52.6
16	160.5	159.0	159.5	160.5	159.7	158.6
17	106.8	107.2	107.1	107.1	106.6	107.1
18	68.3	171.3			67.6	171.8
19	182.0	175.6	179.3	178.9	181.7	175.8
20	21.2	20.4	19.1	19.7	20.3	22.3
OMe		53.4				53.4

* Determined in $[^2H_5]$pyridine.

The importance of the 7-hydroxy group in the oxidative cleavage of ring B was investigated by feeding 18-hydroxykaurenolide (10), which lacks this group. The first time that this experiment was carried out, the metabolites were separated into acidic and neutral fractions. However both fractions gave the same neutral norkaurenolide, $C_{19}H_{26}O_3$ (11). Hence when the experiment was repeated, the extract was methylated with diazomethane prior to chromatography. A monohydroxy-methyl ester, $C_{21}H_{28}O_5$ (12), was then isolated. The location of the new hydroxy group at the 11α-position was established by examination of the ^1H and ^{13}C n.m.r. spectra. Compared to the parent kaurenolide, the ^{13}C n.m.r. spectrum (see Table) lacked a high-field triplet signal at δ 17 p.p.m. which could be assigned to C-11.[17] Instead, there was a new doublet signal at δ 65 p.p.m. In addition the resonances assigned to C-9 and C-12 showed a downfield shift indicative of an adjacent hydroxy group. The stereochemistry of the hydroxy group at position 11 was assigned by examination of the pyridine-induced shift of the C-20 methyl group signal ($\Delta\delta_H$ 0.53 p.p.m.). Hence the parent metabolite was the 11α-hydroxy-18-oic acid (13). The nor-compound showed the same low-field ^1H n.m.r. signal for the angular methyl group. Since it could be prepared from the ester (12) by hydrolysis with sodium carbonate and heating the product, it was assigned the structure (11). A second minor product was also isolated as its methyl ester, $C_{21}H_{28}O_5$. Its ^1H n.m.r. spectrum indicated that it was a secondary alcohol. Since there were no changes in the ring-B C*H*–O or ring D resonances, it is possible that the hydroxy group is

located on ring A. The position of the C*H*OH resonance (δ 3.78) and that of the 5-H (δ 2.31) are comparable with those of 1β,7β-dihydroxykaurenolide.[10] The presence of significant ions at *m/e* 121 (unhydroxylated rings C and D) [10] and 91 and 93 (ring A) in the mass spectrum, lend some support to this, and hence the parent metabolite may be a 1β-hydroxy-18-oic acid. Again no compounds of the fujenal type were detected.

The following conclusions may be drawn from this. 7-Hydroxykaurenolide (3) appears to be a branching point in the metabolic pathways, cleavage of ring B on the one hand affording fujenal, and on the other hand hydroxylation at C-18 leading to the alcohol and thence to the acid. In this pathway further hydroxylation at C-11 can also occur, particularly when the cleavage of ring B is precluded. Furthur metabolism of 7-deoxy-18-hydroxykaurenolide does not appear to lead to insertion of a 7-hydroxy group as a major pathway. The aldehyde anhydride fujenal (4) may be formed by the oxidative cleavage of ring B of a kaurenolide, and also from a 6β,7β-diol (14),[2,18] *i.e.* the insertion of an oxygen atom at the 6β-position may either precede or follow the formation of a 19-6α-lactone ring. In fujenal it is significant that whilst C-6 occurs at the oxidation level of a carboxylate, C-7 is at an aldehyde oxidation level. The oxidative cleavage of ring B bears a formal relationship to the mechanism of the oxidative ring contraction in gibberellin biosynthesis. Ring contraction to form the gibberellins involves the oxidative loss of a 6β-hydrogen and the 7β-hydroxy hydrogen. Dehydrogenation of 6β- and 7β-hydroxy groups in a similar manner could afford a mechanism for the cleavage of ring B. It is possible that the enzyme systems may be related. The inhibitory effect of an 18-hydroxy group on the cleavage of ring B, which is comparable to its effect on ring contraction, may also be understood in terms of its interaction with the oxidant in this scheme.

EXPERIMENTAL

General experimental details have been described previously.[3]

Incubation of 7-*Hydroxykaurenolide* (3) *with* Gibberella fujikuroi.—The fungus was grown on shake culture (100 ml) [3] for 1 d in 15 conical flasks (250 ml) in the presence of 10^{-4}M AMO 1618.

7-Hydroxykaurenolide (3) (140 mg) dissolved in absolute ethanol (14 ml) was distributed between 14 flasks and the remaining flask retained as a control. The incubation was allowed to grow for a further 6 d. The broth was acidified, extracted with ethyl acetate, and separated into acidic and neutral fractions. The neutral fraction was chromatographed on silica. Elution with 15% ethyl acetate–light petroleum afforded the starting material (64 mg). Further elution with 30% ethyl acetate–light petroleum gave *ent*-6β,7α,18-trihydroxykaur-16-en-19-oic acid 19,6-lactone (7,-18-dihydroxykaurenolide) (43 mg), m.p. 212—214 °C (lit.,[8] 211—214 °C), identified by its i.r. and n.m.r. spectra. The acidic fraction was methylated with diazomethane in ether and then chromatographed on silica. Elution with 25% ethyl acetate–light petroleum gave dimethyl *ent*-7-oxo-6,7-secokaur-16-en-6,19-dioate (12 mg), m.p. 141—142 °C, identified by comparison (i.r. and n.m.r.) with an authentic sample.[19]

Incubation of 7,18-*Dihydroxykaurenolide with* Gibberella fujikuroi.—The fungus was grown on shake culture (100 ml) at 25 °C for 1 d in 50 flasks in the presence of 10^{-4}M AMO 1 618. The kaurenolide (0.5 g) in ethanol (48 ml) was distributed between 48 flasks and the remaining two flasks retained as a control. The incubation was allowed to continue for a further 6 d. The broth was acidified, extracted with ethyl acetate, and the extract was separated into acidic and neutral fractions. The neutral fraction afforded the starting material (200 mg) and further starting material was recovered from the mycelium by disruption at −70 °C in acetone–solid carbon dioxide and extraction with ethyl acetate. The acidic fraction was methylated with diazomethane in ether and then chromatographed on silica. Elution with 25% ethyl acetate–light petroleum gave ent-6β,7α-*dihydroxy*-18-*methoxycarbonylkaur*-16-*en*-19-*oic acid* 19,6-*lactone* which was recrystallized from acetone–light petroleum as needles (21 mg), m.p. 183—185 °C (Found: C, 69.7; H, 7.8. $C_{21}H_{28}O_5$ requires C, 70.0; H, 7.8%); $\nu_{max.}$ 3 580, 3 480(br), 1 765, 1 735, 1 650, and 885 cm^{-1}; δ 0.99 (3 H, s, 20-H), 3.74 (3 H, s, OMe), 4.36 (1 H, d, *J* 7 Hz, 7-H), 4.56 (1 H, t, *J* 7 Hz, 6-H), 4.86 and 4.99 (2 H, br d, 17-H); *m/e* 342 (100%, $M^+ - 18$), 315, 314, 297, 283, 267, 195, 133, 121, 119, 107, 105, 95, 93, and 91.

Incubation of 7-*Hydroxy*-18-*norkaurenolide with* Gibberella fujikuroi.—The fungus was grown on shake culture (100 ml) at 25 °C for 1 d in 25 flasks in the presence of 10^{-4}M AMO 1618. The norkaurenolide (200 mg) in ethanol (23 ml) was distributed between 23 flasks and the remaining two flasks were retained as a control. The incubation was allowed to continue for a further 6 d. The broth was acidified, extracted with ethyl acetate, and the extract was separated into acidic and neutral fractions. The neutral fraction was chromatographed on silica. Elution with 30% ethyl acetate–light petroleum gave *ent*-4α,6β,7α-trihydroxy-18-norkaur-16-en-19-oic acid 19,6-lactone (57 mg), which crystallized from ethyl acetate–light petroleum as needles, m.p. 211—213 °C (lit.,[11] 211—213 °C); $\nu_{max.}$ 3 520, 1 768, 1 655, and 885 cm^{-1}; δ 0.80 (3 H, s, 20-H), 1.96 (1 H, d, *J* 7 Hz, 5-H), 4.39 (1 H, d, *J* 7 Hz, 7-H), 4.83 (1 H, t, *J* 7 Hz, 6-H), partly obscured by 4.85 (1 H, br s), and 5.0 (1 H, br s, 17-H_2) (Found: C, 71.3; H, 8.0. Calc. for $C_{19}H_{26}O_4$: C, 71.7; H, 8.2%).

Incubation of ent-6β,18-*Dihydroxykaur*-16-*en*-19-*oic Acid* 19,6-*Lactone with* Gibberella fujikuroi.—The fungus was grown on shake culture (200 ml) at 25 °C for 1 d in 20 flasks (500 ml) in the presence of 10^{-4}M AMO 1 618. The kaurenolide (243 mg) dissolved in ethanol (19 ml), was distributed between 19 flasks and the remaining flask was retained as a control. The incubation was continued for a further 6 d. The broth was separated into acidic and neutral fractions with sodium hydrogencarbonate. The neutral fraction was chromatographed on silica. Elution with 15% ethyl acetate–light petroleum gave ent-6β,11β-*dihydroxy*-18-*norkaur*-16-*en*-19-*oic acid* 19,6-*lactone*, which was recrystallized from acetone–light petroleum as needles (10 mg), m.p. 252—253 °C (Found: C, 74.9; H, 8.6. $C_{19}H_{26}O_3$ requires C, 75.5; H, 8.7%); $\nu_{max.}$ 3 525, 1 760, 1 650, and 885 cm^{-1}; δ 1.23 (3 H, s, 20-H), 4.22 (1 H, m, 11-H), 4.78 (1 H, m, 6-H), and 4.85 and 4.95 (2 H, br d, 17-H). Chromatography of the acidic fraction on silica gave the same norkaurenolide (8 mg), m.p. 252—253 °C, identified by its i.r. and n.m.r. spectra. The incubation was repeated

as above. The broth was extracted with ethyl acetate, rapidly separated into acidic and neutral fractions and the acidic fraction methylated with diazomethane in ether. The product was chromatographed on silica. Elution with 25% ethyl acetate–light petroleum gave ent-6β,11β-*dihydroxy*-18-*methoxycarbonylkaur*-16-*en*-19-*oic acid* 19,6-*lactone*, which was recrystallized from light petroleum as prisms (18 mg), m.p. 176—178 °C (Found: C, 70.1; H, 7.7. $C_{21}H_{28}O_5$ requires C, 70.0; H, 7.8%); $\nu_{max.}$ 3 580, 1 760, 1 725, 1 650, and 885 cm^{-1}; δ 1.22 (3 H, s, 10-H), 3.73 (3 H, s, OMe), 4.22 (1 H, m, 11-H), 4.78 (1 H, m, 6-H), and 4.81 and 4.94 (2 H, br d, 17-H); δ([$^2H_5$]pyridine) 1.55 (3 H, s, 20-H), 4.35 (1 H, m, 11-H), 3.68 (3 H, s, OMe), 4.87 (2 H, m, 6- and 17-H), and 5.01 (1 H, m, 17-H). Further elution gave an unknown kaurenolide (5 mg), which crystallized from light petroleum as prisms, m.p. 143—145 °C (Found: M^+ 360.194. $C_{21}H_{28}O_5$ requires M, 360.194); $\nu_{max.}$ 3 500, 1 760, 1 705, 1 660, and 890 cm^{-1}; δ 0.98 (3 H, s, 20-H), 2.32 (1 H, d, J 7 Hz, 5-H), 3.78 (1 H, m, 1-H), 3.73 (3 H, s, OMe), 4.8 (2 H, m, 6- and 17-H), and 4.96 (1 H, br s, 17-H); *m/e* 360, 345, 342, 333, 301, 298, 255, 239, 187, 145, 121, 119, 107, 93, and 91.

Hydrolysis of ent-6β,11β-*Dihydroxy*-18-*methoxycarbonylkaur*-16-*en*-19-*oic Acid* 19,6-*Lactone*.—The kaurenolide (8 mg) in methanol (1 ml) was heated with saturated aqueous sodium carbonate for 4 h. The solution was acidified, extracted with ethyl acetate, and heated under reflux for 1 h. The solvent was evaporated to afford the norkaurenolide, which was identified by its i.r. spectrum.

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