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Kaurenolides and fujenoic acids are side products of the gibberellin P450-1 monooxygenase in *Gibberella fujikuroi*

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Abstract

The steps involved in kaurenolide and fujenoic acids biosynthesis, from *ent*-kauradienoic acid and *ent*-6α,7α-dihydroxykaurenoic acid, respectively, are demonstrated in the gibberellin (GA)-deficient *Gibberella fujikuroi* mutant SG139, which lacks the entire GA-biosynthesis gene cluster, complemented with the *P450-1* gene of GA biosynthesis (SG139-P450-1). *ent*-[^{2}H]Kauradienoic acid was efficiently converted into 7β-hydroxy[^{2}H]kaurenolide and 7β,18-dihydroxy[^{2}H]kaurenolide by the cultures while 7β-hydroxy[^{2}H]-kaurenolide was transformed into 7β,18-dihydroxy[^{2}H]kaurenolide. The limiting step was found to be hydroxylation at C-18. In addition, SG139-P450-1 transformed *ent*-6α,7α-dihydroxy[$^{14}C_4$]kaurenoic acid into [$^{14}C_4$]fujenoic acid and [$^{14}C_4$]fujenoic triacid. Fujenal was also converted into the same products but was demonstrated not to be an intermediate in this sequence. All the above reactions were absent in the mutant SG139 and were suppressed in the wild-type strain ACC917 by disruption of the *P450-1* gene. Kaurenolide and fujenoic acids synthesis were associated with the microsomal fraction and showed an absolute requirement for NADPH or NADH, all properties of cytochrome P450 monooxygenases. Only 7β-hydroxy[$^{14}C_4$]kaurenolide synthesis and not further 18-hydroxylation was detected in the microsomal fraction. The substrates for the P450-1 monooxygenase, *ent*-kaurenoic acid and [^{2}H]GA_{12}, efficiently inhibited kaurenolide synthesis with I_{50} values of 3 and 6 μM, respectively. Both substrates also inhibited *ent*-6α,7α-dihydroxy[$^{14}C_4$]kaurenoic acid metabolism by SG139-P450-1. Conversely, [$^{14}C_4$]GA_{14} synthesis from [$^{14}C_4$]GA_{12}-aldehyde was inhibited by *ent*-[^{2}H]kauradienoic acid and fujenal with I_{50} values of 10 and 30 μM, respectively. These results demonstrate that kaurenolides and seco-ring B kaurenoids are formed by the P450-1 monooxygenase (GA_{14} synthase) of *G. fujikuroi* and are thus side products that probably result from stabilization of radical intermediates involved in GA_{14} synthesis.

Keywords: *Gibberella fujikuroi*; Biosynthesis; Kaurenolide and fujenoic acids; P450 monooxygenases

1. Introduction

Kaurenolides (diterpenoid lactones **4**, **5** in Fig. 1) and fujenoic acids (e.g. seco-ring B kaurenoids **9**, **10** in Fig. 1) are found in cultures of the gibberellin (GA)-producing fungus *Gibberella fujikuroi* (Cross et al., 1963a,b; Bateson and Cross, 1972; Hedden et al., 1973) (see Fig. 1). These *ent*-kaurenoid derivatives are biosynthetically related to the GAs produced by this fungus and are formed in two branch pathways from the early intermediates of GA biosynthesis, *ent*-kaurenoic acid **1** and *ent*-7α-hydroxykaurenoic acid **6**, respectively (Cross et al., 1970; Bearder et al., 1975; Beale et al., 1982). Kaurenolides (e.g. **4**, **5**) and fujenoic acids (e.g. **9**, **10**) have also been described in plants as by-products of GA biosynthesis (Graebe et al., 1974; Gaskin and MacMillan, 1975; Hedden and Graebe, 1981). 7β-Hydroxykaurenolide **4**, 3β,7β-dihydroxykaurenolide and 7β,18-dihydroxykaurenolide **5** have been identified in cultures of *G. fujikuroi,* (Cross et al., 1963a,b; Bateson and Cross, 1972; Hedden et al., 1973) while 7β,12-dihydroxykaurenolide and 7β,13-dihydroxykaurenolide were found with 7β-hydroxykaurenolide **4** in seeds of *Cucurbita maxima* (Graebe et al., 1974) and *Phaseolus coccineus* (Gaskin and MacMillan, 1975).

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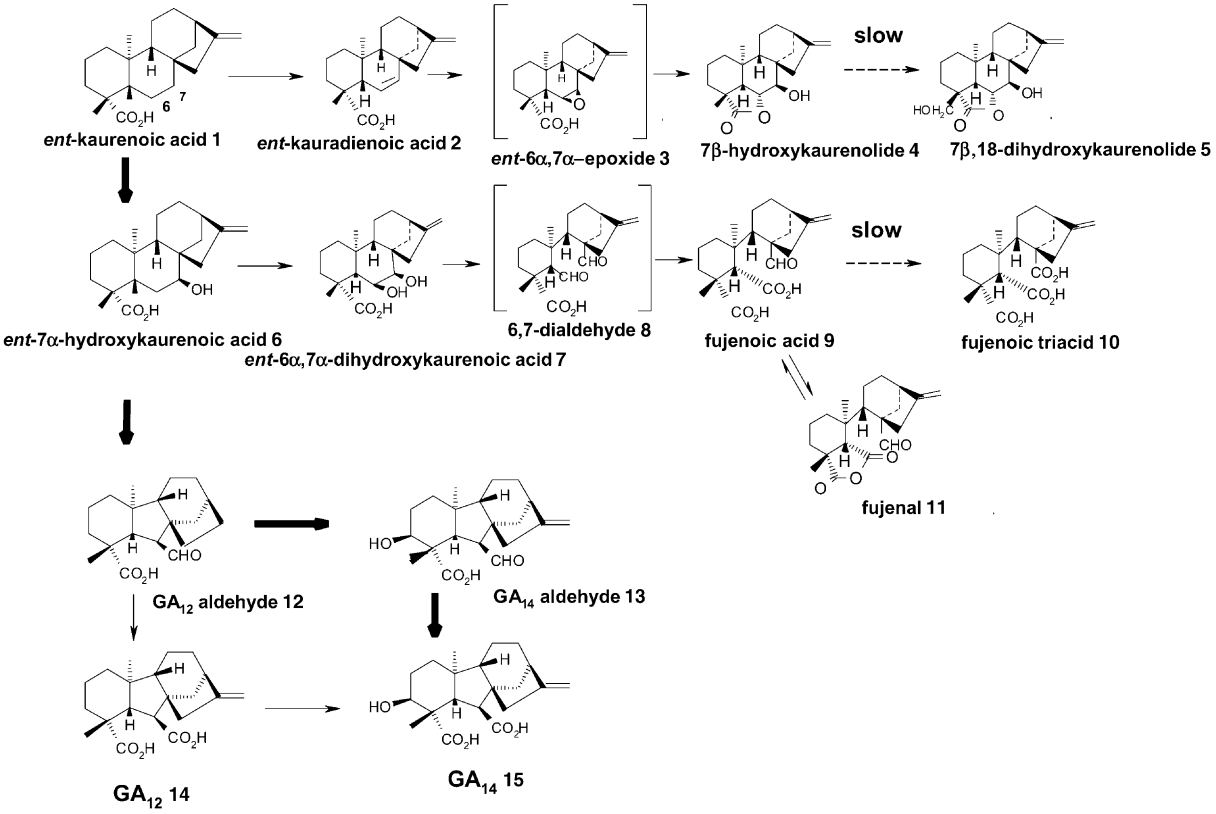

Fig. 1. Reactions in kaurenolide and fujenoic acids biosynthesis, from *ent*-kauradienoic acid **2** and *ent*-6α, 7α-dihydroxykaurenoic acid **7**, demonstrated in SG139-P450-1 and their relationship to the main biosynthetic sequence, from *ent*-kaurenoic acid **1** to GA_{14} **15** catalyzed by the P450–1 monooxygenase (Beale et al, 1982; Rojas et al., 2001). Proposed intermediates (Beale et al., 1982; Hedden, 1983) are shown in brackets. Heavy arrows indicate the proposed major pathway from *ent*-kaurenoic acid **1** to GA_{14} **15**. Dotted arrows indicate slow steps in the branch pathways.

The biosynthetic pathways to kaurenolides and fujenoic acids in *G. fujikuroi* were originally elucidated in precursor administration experiments with labelled precursors added to cultures of wild-type and mutant strains (Cross et al., 1968, 1970; Bearder et al., 1975; Beale et al., 1982). From these studies it was found that the kaurenolides are formed from *ent*-kaurenoic acid **1** through the 6,7-unsaturated intermediate *ent*-kauradienoic acid **2** (Beale et al., 1982), while fujenoic acids (e.g. **9**), kaurenoid compounds containing an oxidized opened ring B, are synthesized from *ent*-7α-hydroxykaurenoic acid **6** through *ent*-6α,7α-dihydroxykaurenoic acid **7** (Cross et al., 1970). Similar results were found in experiments with homogenates of *C. maxima* endosperm which demonstrated conversion of *ent*-kaurenoic acid **1** through *ent*-kauradienoic acid **2** into 7β-hydroxykaurenolide **4** and 7β,12-dihydroxykaurenolide (Hedden and Graebe, 1981). The branch pathway to *ent*-6α,7α-dihydroxykaurenoic acid **7** has been also shown to occur in plants (Graebe et al., 1974,1975).

A detailed study of kaurenolide biosynthesis in *G. fujikuroi* used resuspension cultures of the mutant B1-41a, in which GA biosynthesis was blocked at the *ent*-kaurene oxidase stage (Beale et al., 1982). By incubating unlabelled and labelled potential intermediates it was shown that *ent*-kauradienoic acid **2** forms an *ent*-6α,7α-epoxide **3** to give 7β-hydroxykaurenolide **4**, which is further hydroxylated to 7β,18-dihydroxykaurenolide **5**. It was shown that *ent*-6α,7α-dihydroxykaurenoic acid **7**, which had been postulated as a precursor of kaurenolides, was not an intermediate in their formation (Beale et al., 1982). Fujenal **11** and fujenoic triacid **10** have been demonstrated to be formed in fungal cultures by oxidation of *ent*-6α,7α-dihydroxykaurenoic acid **7**, an intermediate in the branch pathway from *ent*-7α-hydroxykaurenoic acid **6** (Cross et al., 1970, Hanson et al., 1972). By using ^{14}C-labelled precursors and isotopic dilution, *ent*-6α,7α-dihydroxykaurenoic acid **7** was shown to be formed from *ent*-7α-hydroxykaurenoic acid **6** and converted into fujenal **11** (Cross et al., 1970). Even when fujenal **11**, fujenoic acid **9** and fujenoic tracid **10** have been found in cultures of the fungus (Cross et al., 1963a; Fernández-Martin et al., 1995) and in precursor administration experiments (Cross et al., 1970; Rojas et al., 2001) their metabolic relationship is unclear.

Although there is strong evidence that kaurenolides and fujenoic acids are formed by oxidation of *ent*-kaurenoid intermediates in GA biosynthesis, the nature of the enzyme(s) responsible for their synthesis has not

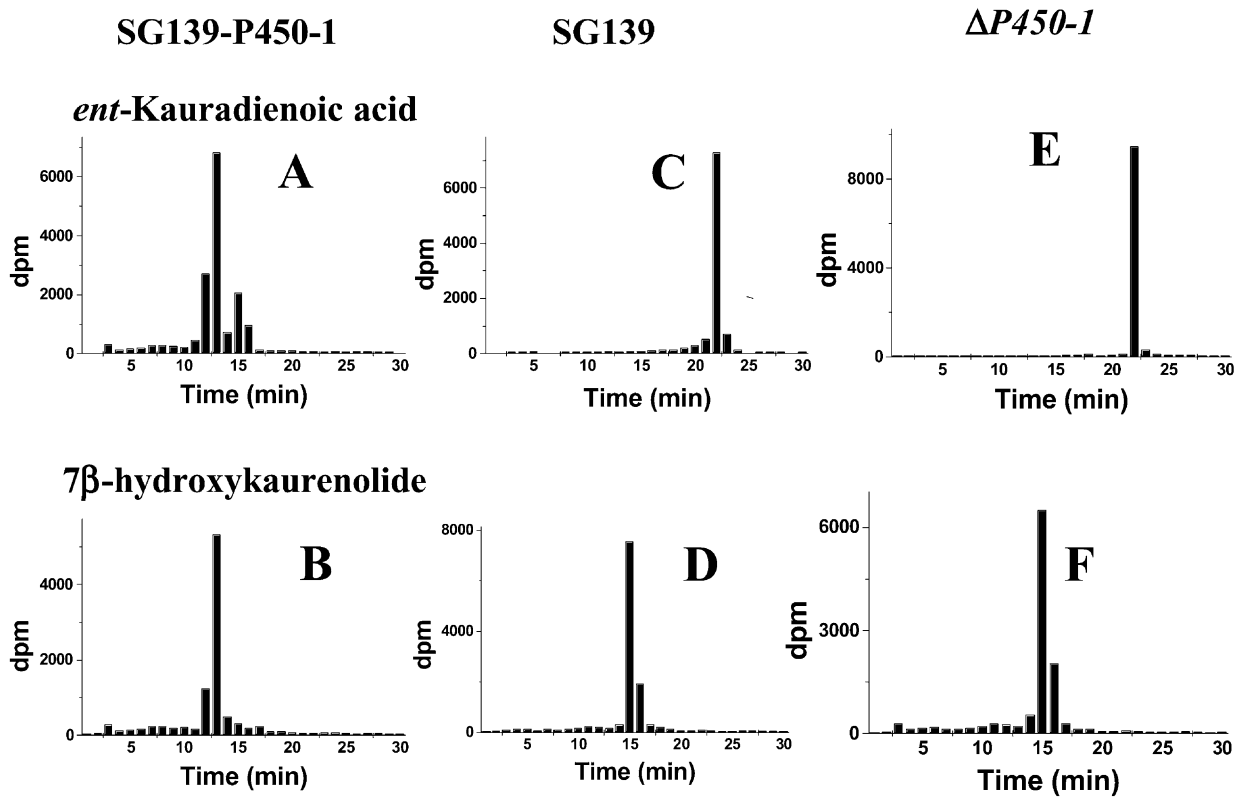

Fig. 2. HPLC radioactivity profiles of total extracts from incubations of *ent*-[$^{14}C_4$]kauradienoic acid **2** (A,C,E) and 7β-hydroxy[$^{14}C_4$] kaurenolide **4** (B,D,F) with cultures of the *G. fujikuroi P450-1* transformant SG139-T7 (A,B), deletion mutant SG139 (C,D) and disruption mutant ΔP450-1 T35 (E,F). Retention times for *ent*-[$^{14}C_4$]kauradienoic acid **2**, 7β-hydroxy-[$^{14}C_4$]kaurenolide **4** and 7β,18-dihydroxy-[$^{14}C_4$]kaurenolide **5** are 22, 15 and 13 min respectively.

been clearly established. We have previously proposed that these products would be generated by the fungal P450-1 monooxygenase that oxidizes *ent*-kaurenoic acid **1** through *ent*-7α-hydroxykaurenoic acid **6** to GA_{14} **15** in gibberellic acid synthesis (Rojas et al., 2001; Hedden et al., 2002). However, it is also possible that they are formed by unspecific oxidases located outside the GA-biosynthesis gene cluster. The P450-1 monooxygenase is encoded by one of the four cytochrome P450 genes (*P450-1–P450-4*) present in the GA gene cluster in *G. fujikuroi* (Tudzynski and Hölter, 1998; Rojas et al., 2001; Hedden et al., 2002) and catalyzes four sequential steps in GA biosynthesis, from *ent*-kaurenoic acid **1** to GA_{14} **15** (Rojas et al., 2001). This enzyme displays a unique multifunctionality catalyzing oxidation at different carbon atoms: C-3, C-6 and C-7. We have suggested that P450-1 may also be involved in the formation of kaurenolides and fujenoic acids, because cultures of the deletion mutant SG139, which lacks the gene cluster, complemented with the *P450-1* gene gave low amounts of labelled 7β-hydroxykaurenolide **4**, 7β,18-dihydroxykaurenolide **5**, 3β,7β-dihydroxykaurenolide and fujenoic acids (e.g. **9**) when incubated with *ent*-17-[^{2}H]kaurenoic acid **1**. Furthermore, this transformant converted *ent*-6α,7α-dihydroxy[$^{14}C_4$]kaurenoic acid **7** into fujenoic acids e.g. **9** (Rojas et al., 2001). All of these reactions involve oxidations on C-6, C-7, C-18 or C-3, consistent with the demonstrated activities of P450-1 in GA biosynthesis (Rojas et al., 2001).

In this paper, we report further evidence to demonstrate that P450-1 is indeed responsible for kaurenolide and fujenoic acids synthesis, which confirms the remarkable substrate versatility of this oxidase. Each of the metabolic steps in both branch pathways were investigated in detail in SG139–P450-1 transformants, as well as in deletion and disruption mutants. This, together with the properties found for the reactions of kaurenolide and fujenoic acid synthesis clearly demonstrate that these kaurenoid products are formed by P450-1 in *G. fujikuroi*.

2. Results and discussion

2.1. Kaurenolide and fujenoic acid synthesis in cultures of SG139–P450-1

Cultures of the deletion mutant SG139, complemented with the *P450-1* gene (SG139–P450-1, transformants T7 and T10) metabolized *ent*-[$^{14}C_4$]kauradienoic acid **1**, the first committed intermediate in kaurenolide biosynthesis, efficiently into two more polar products

Table 1
Kaurenolide and fujenoic acids synthesis in cultures of SG139–P450-1

Substrate	Products	*m/z* (% Relative intensity)[a]
ent-[7-$^2H_1$]Kauradienoic acid **2**	7β-Hydroxy-[^{2}H]kaurenolide **4** (22%)[b]	389(2), 346(8), 299(60), 284(11), 271(13), 256(8), 243(4), 228(8), 205(7), 163(13), 145(23), 137(100), 109(63)
	7β-Hydroxykaurenolide **4** (library spectrum)	M^+ 388(2), 345(11), 298(100), 283(11), 270(25), 255(11), 242(8), 227(9), 205(10), 163(17), 145(20), 137(98), 109(49)
	7β,18-Dihydroxy-[^{2}H]kaurenolide **5** (78%)	462(2), 387(53), 359(5), 343(4), 297(17), 284(62), 269(100), 254(15), 226(13)
	7β,18-Dihydroxy-kaurenolide **5** (library spectrum)	M^+ 476(1), 461(3), 433(2), 386(32), 358(8), 343(9), 296(24), 283(53), 268(100), 253(7), 225(9)
7β-Hydroxy7α-[$^2H_1$]kaurenolide **4**	7β,18-Dihydroxy-[^{2}H]kaurenolide **5** (95%)	462(4), 434(2), 387(63), 359(7), 343(8), 297(16), 284(60), 269(100), 254(23), 225(15)
ent-6α,7α-Dihydroxy-[$^{14}C_4$]kaurenoic acid **7**	[$^{14}C_4$]Fujenoic acid **9** (90%)	231(8), 227(8), 199(29), 195(28), 171(26), 167(28), 139(18), 135(15), 113(19), 111(90),109(49), 107(100) [KRI[c] 2537]
	Fujenoic acid **9** (library spectrum)	M^+ 376(1), 358(3), 345(5), 316(8), 301(6), 284(5), 257(5), 239(6), 227(64), 195(90), 167(100), 135(20), 109(33), 107(81) [KRI 2530]
	[$^{14}C_4$]Fujenoic triacid **10** (10%)	231(7), 227(8), 199(33), 195(40), 171(18), 167(21), 139(24), 135(16), 111(89), 107(100) [KRI 2545]
	Fujenoic triacid **10** (library spectrum)	M^+ 410(1), 375(2), 342(2), 315(3), 283(2), 255(4), 227(56), 195(100), 167(70), 135(7), 107(75) [KRI 2541]
Fujenal **11**	Fujenoic acid **9** (60%)[d]	M^+ 492(2), 477(23), 402(6), 343(71), 330(6), 299(7), 253(80), 241(15), 225(28), 181(23), 147(48), 135(38), 109(100), 107(89)
	Fujenoic triacid **10** (30%)[d]	M^+ 580(4), 565(38), 343(100), 328(7), 299(5), 253(60), 225(13), 181(13), 147(38), 109(33), 107(43)
ent-[$^{14}C_4$]Kaurenoic acid **1**	[^{14}C]GA_{14} **15** (78%)[e]	
[$^{14}C_4$]GA_{12}-aldehyde **12**	[^{14}C]GA_{14} **15** (83%)[e]	

[a] For ions > *m/z* 100.
[b] Percent conversion is shown in parenthesis for each product.
[c] KRI, Kovats Retention Index.
[d] Analysed as TMS esters.
[e] Identified by the retention time on HPLC. The GC–MS analysis of this product has already been described (Rojas et al., 2001).

with retention times (Rt) of 13 min and 15 min in HPLC (Fig. 2A). The products were identified by GC–MS, after separation from an incubation with *ent*-[^{2}H]kauradienoic acid **2** (232 nmol) and *ent*-[$^{14}C_4$]kauradienoic acid **2** (200,000 dpm, 0.42 nmoles), as 7β-hydroxy[^{2}H]-kaurenolide **4** (Rt 15 min) and 7β,18-dihydroxy[^{2}H]-kaurenolide **5** (Rt 13 min) (Table 1). Both products as well as 3β,7β-dihydroxy[^{2}H]kaurenolide had been reported as minor products in incubations of mg amounts of *ent*-17-[^{2}H]kaurenoic acid **1** with cultures of SG139–P450-1 (Rojas et al., 2001). In contrast, complete conversion of *ent*-[$^{14}C_4$]kauradienoic acid **2** to [$^{14}C_4$]kaurenolides by the *P450-1* transformants was found (Fig. 2A) as expected for an intermediate that participates exclusively in this branch pathway and does not compete with the reactions of GA_{14} **15** synthesis. Incubations of SG139–P450-1 with low amounts of *ent*-[$^{14}C_4$]kaurenoic acid **1** (18 ng, Table 1) gave no detectable kaurenolides or fujenoic acids as products, confirming that these are formed in minor side reactions from this GA precursor.

7β-hydroxy[^{2}H]kaurenolide **4** was purified from the above reaction media and re-incubated with cultures of SG139-P450-1, which converted it completely to 7β,18-dihydroxy[^{2}H]kaurenolide **5** (Table 1). This reaction was demonstrated also with 7β-hydroxy[$^{14}C_4$]kaurenolide **4** (Fig. 2B). We did not detect 3β, 7β-dihydroxykaurenolide under these conditions. Thus, all the steps involved in kaurenolide synthesis and previously described in *G. fujikuroi* (Beale et al., 1982) are present in SG139–P450-1 transformants. In contrast, *ent*-[$^{14}C_4$]-kauradienoic acid **2** and 7β-hydroxy[^{14}C]kaurenolide **4** remained unchanged when incubated with the deletion mutant SG139 (Fig. 2 C,D), suggesting that the P450-1 monooxygenase is responsible for the oxidation reactions detected in the SG139–P450-1 transformants.

The time course of *ent*-[$^{14}C_4$]kauradienoic acid **2** metabolism (Fig. 3) revealed marked differences in the relative rates of the two steps in 7β, 18-dihydroxy[^{14}C]-kaurenolide **5** synthesis. A 74% conversion of the substrate into 7β-hydroxy[^{14}C]kaurenolide **4** was obtained after 10 min incubation and almost complete transformation (90%) into this intermediate was found within 30 min. In contrast, the concentration of 7β,18-dihydroxy[^{14}C]kaurenolide **5** began to increase in the reaction media only after 5 h of incubation with 57% conversion obtained after 17.5 h. Thus, hydroxylation at position 18 is rate-limiting in the reaction sequence to 7β,18-dihydroxy[^{14}C]kaurenolide **5** suggesting that carbon-18 in the intermediate 7β-hydroxykaurenolide **4** would be less accessible to the reaction centre of P450-1 than C-6 and C-7 of *ent*-kauradienoic acid **2** in the former

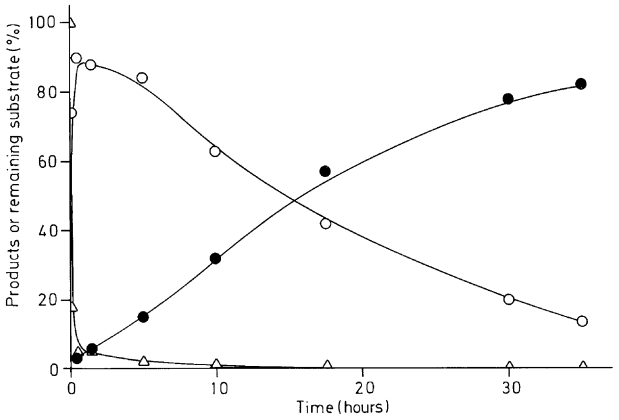

Fig. 3. Time course of *ent*-[$^{14}C_4$]kauradienoic acid **2** metabolism by cultures of SG139–P450-1. The products, 7β-hydroxy[$^{14}C_4$]kaurenolide **4** (○) and 7β, 18-dihydroxy-[$^{14}C_4$]kaurenolide **5** (●) and remaining substrate (Δ) were quantified in the reaction media after extraction and separation by HPLC. Conditions in Section 3.

reaction. Low binding of 7β-hydroxykaurenolide **4** to the enzyme could also account for this result. Moreover, 18-hydroxylation occurs only with 7β-hydroxykaurenolide **4** and not with *ent*-kauradienoic acid **2** or any other tested substrate, suggesting that a change in substrate binding orientation takes place after 7β-hydroxykaurenolide **4** synthesis. A change in substrate binding orientation has also been suggested for the oxidation sequence from *ent*-kaurenoic acid **1** to GA_{14} **15** catalyzed by P450-1 in which oxidations at C-7 and C-6 (ring B contraction) precede the 3β-hydroxylation reaction (Rojas et al., 2001).

Cultures of SG139–P450-1 converted *ent*-6α,7α-dihydroxy[$^{14}C_4$]kaurenoic acid **7** completely to more polar products which were purified by TLC and then identified by GC-MS as [^{14}C]fujenoic acid **9** and [^{14}C]fujenoic triacid **10** (Table 1). Even when complete conversion of the precursor was obtained, [^{14}C]fujenoic acid **9** accumulated in the reaction medium. There was no conversion of *ent*-6α,7α-dihydroxy[$^{14}C_4$]kaurenoic **7** acid by the SG139 deletion mutant. After incubating unlabelled fujenal **11** with cultures of SG139–P450-1, fujenoic acid **9** and fujenoic triacid **10** were recovered in a ratio of approximately 3:2 (Table 1), whereas fujenoic acid **9** with approximately 10% triacid were recovered from incubations of fujenal **11** with SG139 (not shown) suggesting that P450-1 is also responsible for the oxidation of fujenal acid **9** to the triacid **10**. The small amount of triacid formed in the absence of P450-1 could result from non-enzymatic oxidation or action of nonspecific oxidases.

Although *ent*-6α,7α-dihydroxykaurenoic acid **7** has been shown to be formed from *ent*-7α-hydroxykaurenoic acid **6** in cultures of *G. fujikuroi* (Cross et al., 1970) and also in cell-free systems from *Cucurbita maxima* endosperm (Graebe and Hedden, 1974), we could not detect this transformation in cultures of SG139–P450-1 probably because of efficient oxidation of *ent*-7α-hydroxykaurenoic acid **6** to GA_{12}-aldehyde **12** by the P450-1 monooxygenase. The side reaction to *ent*-6α,7α-dihydroxykaurenoic acid **7** was apparently unable to compete with the main oxidation sequence at the substrate concentrations used. Nevertheless, the finding of efficient conversion of *ent*-6α,7α-dihydroxykaurenoic acid **7** and fujenal **11** into fujenoic acids in SG139–P450-1 indicates strongly that P450-1 activity is responsible for the branch pathway to seco-ring B products in the fungal system.

The involvement of the P450-1 monooxygenase in kaurenolide and fujenoic acid **9** synthesis was further supported by the suppression of these reactions upon disruption of the *P450-1* gene in the wild-type strain ACC917 (Fig. 2E,F). No conversion of *ent*-[$^{14}C_4$]kauradienoic acid **2** or 7β-hydroxy[$^{14}C_4$]kaurenolide **4** was found in cultures of the disruption mutant ΔP450-1 T35 in which the *P450-1* locus was inactivated by transformation with a linearized replacement vector (Rojas et al., 2001). Both kaurenolide precursors remained unchanged in ΔP450-1 cultures, in contrast to their complete metabolism by SG139–P450-1. The fujenoic acids precursor *ent*-6α,7α-dihydroxy[$^{14}C_4$]kaurenoic acid **7** was not metabolized by the wild-type strain after disruption of the *P450-1* gene (not shown).

2.2. Intermediates in fujenoic triacid *10* synthesis

The above results indicate that the synthesis of fujenoic acid **9** and triacid **10** is catalyzed by P450-1. Fujenal **11** is the anhydride of fujenoic acid **9** and may be an artifact, formed from fujenoic acid **9** during extraction and purification (Bearder, 1983). In fact we found fujenal in some but not all incubations with *ent*-6α,7α-dihydroxykaurenoic acid **7** whereas fujenoic acid **9** was always formed. In order to obtain further evidence about the oxidation sequence to fujenoic triacid **10** and to determine if fujenal **11** is formed in vivo, we incubated *ent*-6α,7α-dihydroxy[$^{14}C_4$]kaurenoic acid **7** with cultures of SG139–P450-1 in the presence of variable amounts of unlabelled fujenal **11** (Table 2). ^{14}C was detected in the recovered fujenoic acid **9** and triacid **10** with similar incorporation, but no ^{14}C was found in fujenal **11**. It is, therefore, unlikely that fujenal **11** was formed in vivo, and, furthermore, it was apparently not formed from fujenoic acid **9** under our extraction and work-up conditions. The results also indicate that fujenoic triacid **10** is formed from the diacid (fujenoic acid **9**), and not another, unidentified intermediate, due to the action of P450-1. The relative amounts of products formed at different substrate ratios in the above experiment show that fujenoic acid **9** accumulates in the reaction media which suggests that the oxidation step from fujenoic acid **9** to the triacid **10** would be rate-limiting in this branch pathway. The formation of fujenoic acid **9** from *ent*-6α,7α-dihydroxykaurenoic acid **7** requires two

Table 2
Metabolism of *ent*-6α,7α-dihydroxy[$^{14}C_4$]kaurenoic acid **7** in the presence of variable amounts of fujenal **11**

Substrates (nmol)[a]	Relative amounts of products[b]			
ent-6α,7α-Dihydroxy-[$^{14}C_4$]kaurenoic acid **7**	Fujenal **11**	Fujenal **11**	Fujenoic acid **9**	Fujenoic triacid **10**
1.3	0.63	1.0	3.0 (0.15)	2.0 (0.19)
1.3	1.3	1.0	2.1 (0.21)	1.3 (0.13)
1.3	2.6	1.0	1.6 (0.09)	1.2 (0.08)
0	10	1.0	8.1 (0)	5.3 (0)

[a] 300,000 dpm of *ent*-6α,7α-dihydroxy[$^{14}C_4$]kaurenoic acid **7** (1.3 nmol) and variable amounts of unlabelled fujenal **11** were incubated with cultures of SG139–P450-1. Incubation conditions in Section 3.
[b] Analyzed by GC–MS. The $^{14}C/^{12}C$ ratio in the products is shown in parentheses.

oxidation steps; a single oxidation step would produce the 6, 7-dialdehyde **8** (Hedden, 1983). However, this intermediate has not been detected in *G. fujikuroi* cultures or in our incubations, and is probably too rapidly oxidized to fujenoic acid **9** to accumulate to detectable levels.

2.3. Characterization of the reactions involved in kaurenolide and fujenoic acids synthesis

A microsomal fraction was obtained from the mycelia of SG139-P450-1 by centrifugation at 100,000 *g* and incubated with precursors of kaurenolide and fujenoic acids synthesis. *ent*-[$^{14}C_4$]Kauradienoic acid **2** and *ent*-6α,7α-dihydroxy[$^{14}C_4$]kaurenoic acid **7** were metabolised by this fraction in the presence of NADPH and FAD (Table 3). Both precursors remained unchanged when incubated under the same conditions with the soluble fraction (S100, Table 3) which indicates that kaurenolides and fujenoic acids are formed by membrane-bound oxidase(s).

The microsomal enzymatic activities responsible for kaurenolide and fujenoic acid synthesis required pyridine nucleotides as cofactors (Table 4). FAD was found to activate these reactions (not shown) and thus was included in the reaction media in addition to the nucleotides. Activation by FAD has already been described for 7-oxidase and 3β-hydroxylase activities of the P450-1 monooxygenase and attributed to dissociation of this cofactor from the enzyme during preparation of the microsomes (Urrutia et al., 2001). No activity was obtained in the absence of cofactors in contrast to almost complete (91%) conversion of *ent*-[$^{14}C_4$]kauradienoic acid **2** into 7β-hydroxy-[$^{14}C_4$]kaurenolide **4** in the presence of 1 mM NADPH plus 5 μM FAD. Interestingly, the dihydroxylated product 7β, 18-dihydroxy[$^{14}C_4$]kaurenolide **5** was not detected in our microsomal incubations, probably due to partial loss of enzyme activity upon cell disruption. This would make the rate limiting step in kaurenolide biosynthesis, 18-hydroxylation of 7β-hydroxy[$^{14}C_4$]kaurenolide **4**, no longer detectable. Similarly, it was found previously that microsomes of SG139–P450-1 failed to convert [$^{14}C_4$]GA_{12} **12** into [$^{14}C_4$]GA_{14} **15**, a reaction easily detected in cultures (Rojas et al., 2001). Conversion of *ent*-[$^{14}C_4$]kauradienoic acid **2** into 7β-hydroxy[$^{14}C_4$]kaurenolide **4** was also obtained in the presence of 1 mM NADH and 50 μM FAD (Table 4) although at a lower rate (37% conversion). The conversion of *ent*-6α,7α-dihydroxy[$^{14}C_4$]kaurenoic acid **7** to [$^{14}C_4$]fujenoic acids (e.g. **9**) by the microsomes had the same cofactor requirement as kaurenolide formation with both NADPH and, less efficiently, NADH effective (Table 4). These results are consistent with the participation of a cytochrome P450 monooxygenase in fujenoic acids and kaurenolide synthesis and agree with the nucleotide specificity described for 7-oxidase activity by P450-1 (Urrutia et al., 2001). In contrast, the 3β-hydroxylase activity of P450-1, measured with [$^{14}C_4$]GA_{12}-aldehyde **12** as substrate, has been found to depend absolutely on NADPH (Urrutia et al., 2001). We investigated further, using the microsomes of SG139–P450-1, the cofactor requirement of the different steps of [$^{14}C_4$]GA_{14} **15** synthesis from *ent*-[$^{14}C_4$]kaurenoic acid **1** and ent-7α-hydroxy-[$^{14}C_4$]kaurenoic acid **6**, and confirmed that both substrates are metabolized in the presence of NADPH or NADH as reductants (Table 4). The products formed are, however different, with only 7-oxidase activity and the ring B contraction reaction, which is initiated by oxidation at C-6, detected with NADH,

Table 3
Distribution of enzymatic activities involved in kaurenolide and fujenoic acid synthesis

Fraction[a]	Volume (ml)	7β-Hydroxy[$^{14}C_4$] kaurenolide **4** synthesis (nkat×10^5)[b]	[$^{14}C_4$]Fujenoic acids **9** synthesis (nkat×10^5)[c]
S10	9.5	570	265
P100	0.8	450	209
S100	9.2	5	3

[a] S10: 10,000 g supernatant; P100: 100,000 g pellet; S100: 100,000 g supernatant.
[b] Assayed with *ent*-[$^{14}C_4$]kauradienoic acid **2** in the presence of 1 mM NADPH and 5 μM FAD.
[c] Assayed with *ent*-6α,7α-dihydroxy[[$^{14}C_4$]kaurenoic acid **7** in the presence of 1 mM NADPH and 5 μM FAD.

Table 4
Cofactor requirement for reactions of the branch pathways to kaurenolides and fujenoic acids and for GA_{14} **15** synthesis, in microsomes of SG139–P450-1

Substrate	Cofactors[a]	Products (% conversion)[b]
ent-[$^{14}C_4$]Kauradienoic acid **2**	None	–[c]
	NADPH/FAD	7β-Hydroxy[$^{14}C_4$]kaurenolide **4** (91%)
	NADH/FAD	7β-Hydroxy[$^{14}C_4$]kaurenolide **4** (37%)
ent-6α,7α-Dihydroxy-[$^{14}C_4$]kaurenoic acid **7**	None	–[c]
	NADPH/FAD	[$^{14}C_4$]Fujenoic acids **9**, **10** (83%)
	NADH/FAD	[$^{14}C_4$]Fujenoic acids **9**, **10** (15%)
ent-[$^{14}C_4$]Kaurenoic acid **1**	None	–[c]
	NADPH/FAD	[$^{14}C_4$]GA_{14} **15** (68%)
		ent-7α-Hydroxy[$^{14}C_4$]kaurenoic acid **6** (25%)
		[$^{14}C_4$]GA_{12} **14** (7%)
	NADH/FAD	*ent*-7α-Hydroxy[$^{14}C_4$]kaurenoic acid **6** (26%)
		[$^{14}C_4$]GA_{12} **14** (5%)
ent-7α-Hydroxy[$^{14}C_4$]kaurenoic acid **6**	None	–[c]
	NADPH/FAD	[$^{14}C_4$]GA_{14} **15** (77%)
		[$^{14}C_4$]GA_{12} **14** (10%)
	NADH/FAD	[$^{14}C_4$]GA_{12} **14** (21%)
[$^{14}C_4$]GA_{12} aldehyde **12**	None	–[c]
	NADPH/FAD	[$^{14}C_4$]GA_{14} **15** (63%)
		[$^{14}C_4$]GA_{12} **14** (24%)
	NADH/FAD	[$^{14}C_4$]GA_{12} **14** (39%)

[a] 1 mM NADPH and 5 μM FAD or 1 mM NADH and 50 μM FAD were added as cofactors. 10 or 50 μl of enzyme were utilized in assays with NADPH or NADH, respectively.
[b] Products were quantified after separation in HPLC. Conditions in Section 3. The mixture of fujenoic acid **9** and triacid **10** was quantified after separation from the substrate by TLC.
[c] No products and only residual substrate was detected.

while 3β-hydroxylation was found in addition to these activities in the presence of NADPH. These results confirm the absolute requirement of the GA 3β-hydroxylase activity for NADPH and show that the reactions of kaurenolide and fujenoic acid synthesis have the same cofactor requirements as the oxidation reactions at C-6 and C-7, catalyzed by P450-1. In general, all the oxidation reactions detected in the presence of NADH in SG139–P450-1 microsomes occur at lower rates than with NADPH (Table 4).

2.4. Inhibition of kaurenolide and fujenoic acid synthesis by substrates of the GA_{14} synthase (P450-1)

Unlabelled *ent*-kaurenoic acid **1** as well as [^{2}H]GA_{12} **14**, substrates of P450-1 monooxygenase, were effective inhibitors of 7β-hydroxy[$^{14}C_4$]kaurenolide **4** synthesis from *ent*-[$^{14}C_4$]kauradienoic acid **2** (Table 5) with I_{50} values of 3 and 6 μM, respectively. Both these substrates of GA_{14} synthase also inhibited [$^{14}C_4$]fujenoic acids synthesis from *ent*-6α,7α-dihydroxy[$^{14}C_4$]kaurenoic acid **7** with I_{50} values of 30 μM and 75 μM, respectively. Conversely, *ent*-[^{2}H]kauradienoic acid **2** and fujenal **11** inhibited [$^{14}C_4$]GA_{14} **15** synthesis from *ent*-[$^{14}C_4$]kaurenoic acid **1** or [$^{14}C_4$]GA_{12}-aldehyde **12** by the microsomes (Table 6). These inhibition results are consistent with binding of the precursors of kaurenolide and fujenoic acids synthesis to the P450-1 monooxygenase and thus, together with the results described above on the properties and localization of these reactions confirm our preliminary suggestion about the involvement of P450-1 in the two branches to the kaurenolides and fujenoic acids from the main GA-biosynthetic pathway (Fig. 1). *ent*-Kaurenoic acid **1** would thus be the common precursor of GAs, kaurenolides and seco-ring B kaurenoids through oxidations at C-6 and C-7 catalyzed

Table 5
Inhibition of kaurenolide and fujenoic acids synthesis by the substrates of GA_{14} synthase (P450-1)

Substrate[a]	Inhibitor	I_{50} (μM)
ent-[$^{14}C_4$]Kauradienoic acid **2**	*ent*-Kaurenoic acid **1**	3
	[^{2}H]GA_{12} **14**	6
ent-6α,7α-Dihydroxy-[$^{14}C_4$]kaurenoic acid **7**	*ent*-Kaurenoic acid **1**	30
	[^{2}H]GA_{12} **14**	75

[a] Assay conditions as in Section 3 with 1 mM NADPH and 5 μM FAD as cofactors.

Table 6
Inhibition of GA_{14} synthesis by precursors of kaurenolides and fujenoic acids

Substrate[a]	Inhibitor	I_{50} (μM)
ent-[$^{14}C_4$]Kaurenoic acid **1**	*ent*-[^{2}H]Kauradienoic acid **2**	43
	Fujenal **11**	91
[$^{14}C_4$]GA_{12}-aldehyde **12**	*ent*-[^{2}H]Kauradienoic acid **2**	10
	Fujenal **11**	30

[a] Assay conditions as in Section 3 with 1 mM NADPH and 5 μM FAD as cofactors.

by GA_{14} synthase. These reactions include hydroxylation, aldehyde oxidation, desaturation and rearrangement, all characteristic of P450 enzymes (Sono et al., 1996). In addition, the formation of the *ent*-6α, 7α-epoxide **3** from *ent*-kauradienoic acid **2**, which has been reported in 7β-hydroxykaurenolide **4** synthesis (Beale et al., 1982) but was not detected in our experiments, is consistent with the participation of P450-1 since oxidation of unsaturated compounds to epoxides by P450 monooxygenases has been observed (Ostovic and Bruice, 1992). Kaurenolides and fujenoic acids would thus be side products of P450-1 activity, resulting from alternative stabilization of the radical intermediates involved in 7-oxidation and ring B contraction steps in the main sequence to GA_{14}. This would explain the same cofactor requirement found for the synthesis of kaurenolides, fujenoic acids (e.g. **9**) and GA_{12} **14** by the microsomes of SG139–P450-1 (Table 4). Electrons for the generation of these radical intermediates can come from NADPH as well as from NADH, in contrast to the 3β-hydroxylation reaction catalyzed by P450-1 which obtains electrons exclusively from NADPH (Table 4).

In plants, the P450 monooxygenases that catalyze oxidation of *ent*-kaurenoic acid **1** to GA_{12} **14**, *ent*-kaurenoic acid oxidases (KAO) (Helliwell et al., 2001; Davidson et al., 2003) might also be expected to produce kaurenolides and seco-ring B compounds. Indeed, heterologous expression in yeast of *ent*-kaurenoic acid oxidases from pea (PsKAO1 and PsKAO2) (Davidson et al., 2003) has been found to convert *ent*-kaurenoic acid **1** into 7β-hydroxykaurenolide **4** and *ent*-6α,7α-dihydroxykaurenoic acid **7** besides *ent*-7α-hydroxykaurenoic acid **6**, GA_{12}-aldehyde **12** and GA_{12} **14**, the main products of this oxidases. Furthermore, cell-free systems from *C. maxima* (pumpkin) endosperm converts *ent*-kaurenoic acid **1** into kaurenolides (Hedden and Graebe, 1981), and *ent*-7α-hydroxykaurenoic acid **6** into *ent*-6α,7α-dihydroxykaurenoic acid **7** and seco-ring B compounds (Graebe et al., 1974), although the *ent*-kaurenoic acid oxidase has not been isolated from this source so it is not yet known if a single enzyme is responsible for the formation of these metabolites. Thus, by-products of *ent*-kaurenoic acid oxidase activity are formed in plants as well as in fungi, although it is not known if they are formed in plant tissues other than developing seed. Seed may contain *ent*-kaurenoic acid oxidases with low regiospecificity and/or the flux through the GA-biosynthetic pathway is very high.

Finally, our results confirm the remarkable multifunctionality (substrate versatility) of the P450-1 monooxygenase of *G. fujikuroi* that, besides catalyzing six steps in GA_{14} synthesis is responsible for the oxidation reactions involved in the two branch pathways to kaurenolides and fujenoic acids (Fig. 1). The reactions catalyzed by P450-1 involve oxidations at four C atoms: C-7, C-6, C-3 and C-18. Perhaps more remarkably, the electron source for oxidation on C-3 differs from that for the other reactions in that 3-oxidation has a specific requirement for NADPH. Experiments are in progress to further characterize this remarkable feature of the enzyme through examining its interaction with electron transport proteins.

3. Experimental

3.1. Culture conditions

The SG139–P450-1 transformants T7 and T10 of *Gibberella fujikuroi* (Rojas et al., 2001) were utilized for these studies. The deletion mutant SG139 that lacks the GA gene cluster (Barrero et al., 1999) and the disruption mutant ΔP450-1 T35 (Rojas et al., 2001) were used as control strains. Cultures were maintained in potato dextrose agar and used for inoculating into liquid 40% ICI medium (Geissmann et al., 1996). After 3 days the culture was transferred into 0% ICI medium and grown for additional 7 days for enzyme extraction. Liquid cultures were grown at 28° C under light with orbital shaking at 150 rpm.

3.2. Labelled substrates

[$^{14}C_4$]GA_{12}-aldehyde **12**, *ent*-[$^{14}C_4$]kaurenoic acid **1**, ent-7α-hydroxy[$^{14}C_4$]kaurenoic acid **6**, *ent*-[$^{14}C_4$]kauradienoic acid **2** and ent-6α,7α-dihydroxy-[$^{14}C_4$]kaurenoic acid **7** were synthesized from *R*-[2-^{14}C]mevalonic acid (Amersham) by incubation with pumpkin endosperm enzymes and appropriate cofactors as described (Urrutia et al., 2001). *ent*-Kaurenoic acid **1** and [18-^{2}H]GA_{12} **14** were gifts from Professor M. Beale (Rothamsted Research, UK).

3.3. Production of microsomes

Mycelia grown for 7 days in 0% ICI medium (Geissmann et al., 1966) were harvested by vaccum filtration and washed with ultrapure water and 50 mM Tris–HCl, pH 7.5. Washed mycelia (about 700 mg) were suspended

in 10 ml of the extraction solution (50 mM Tris–HCl pH 8.0; 5 mM EDTA; 4 mM DTT; 2.5 M sucrose and 10 mg/ml bovine serum albumin), frozen at −20 °C and disrupted under 28,000 psi in a chilled X-Press (Edebo, 1983). After thawing, the homogenate was centrifuged at 10,000 *g* for 20 min and the supernatant centrifuged at 100,000 *g* for 1 h. The pellet was supended in 50 mM Tris–HCl pH 7.5, 4 mM DTT, 10 mg/ml bovine serum albumin and 2 M sucrose (0.5 ml) and stored at −70 °C.

3.4. Incubations with fungal cultures

Fungal mycelia grown in 40% ICI medium for 3 days were washed and resuspended in 0% ICI medium (Geissmann et al., 1966). One-millilitre aliquots were transferred to 25-ml sterile flasks containing 5 ml of the same solution and radiolabelled substrates (30,000–200,000 dpm per flask) were added as MeOH solutions (10–40 μl) to the cultures. After incubation on an orbital shaker at 28 °C for 2 days, cultures were filtered and products extracted into EtOAc and purified as described (Rojas et al., 2001). HPLC or TLC analyses were made as described below.

3.5. Enzyme assays

Incubations were carried out in a final volume of 100 μl containing the microsomal suspension (10–80 μl), labelled substrate (30,000 dpm, 0.75 μM), oxidase cofactors (NADPH 1 mM plus FAD 5 μM or NADH 1 mM plus FAD 50 μM) and 50 mM Tris–HCl, pH 7.5. Inhibitors were added as methanolic solutions (5–10 μl) in some incubations. The reaction mixture was incubated with shaking for 1 h at 30 °C and the reaction stopped by adding 10 μl of acetic acid and 1 ml of water (acidified to pH 3.0 with acetic acid) into the reaction media. The samples were further purified on C_{18} Bakerbond cartridges (J.T. Baker) and the products analyzed by HPLC (for kaurenolide and GA_{14} synthesis) or TLC (for fujenoic acids synthesis).

3.6. HPLC conditions

Products were separated on a Symmetry C_{18} column (5 μM; 250×4; Waters) in a Waters 600 HPLC instrument. Elution conditions for *ent*-[$^{14}C_4$]kauradienoic acid **2** and [$^{14}C_4$]kaurenolides were: a 15 min gradient from 75 to 100% MeOH/H_2O pH 3.0, followed by 15 min MeOH. A linear gradient from 60 to 100% methanol/H_2O, pH 3.0, over 30 min was used to separate the products formed from incubations with radiolabeled *ent*-kaurenoic acid **1**, *ent*-7α-hydroxykaurenoic acid **6** and GA_{12}-aldehyde **12**. Fractions (1 ml) were collected and the radioactivity measured by liquid scintillation counting.

3.7. TLC conditions

[$^{14}C_4$]Fujenoic acid **9**, **10** synthesis was monitored by TLC on silica gel G plates developed with hexane: EtOAc (3:7) containing drops of formic acid. Radiactivity was determined in the different fractions by liquid scintillation counting. The substrate, 6α,7α-dihydroxy[$^{14}C_4$]kaurenoic acid **7**, has a Rf value of 0.7 under these conditions, while fujenoic acid **9** and triacid **10** migrate together with a Rf value of 0.28.

3.8. Product identification

Radioactive fractions from HPLC or TLC were derivatized and analyzed by combined gas chromatography–mass spectrometry as described, by comparison with authentic gibberellin standards (Gaskin and MacMillan 1992; Urrutia et al., 2001). Fujenoic acids were analyzed as TMSi esters rather than methyl esters to better separate fujenoic acid **9** and triacid **10**.

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References

Bateson, J.H., Cross, B.E., 1972. New metabolites of *Gibberella fujikuroi*. Part XIX. 3β,7β-dihydroxy-kaurenolide. J.Chem.Soc. Perkin I 1117–1120.

Barrero, A.F., Oltra, J.E., Cabrera, E., Reyes, F., Alvarez, M., 1999. Metabolism of gibberellins and *ent*-kaurenoids in mutants of *Gibberella fujikuroi*. Phytochem. 50, 1133–1140.

Beale, M.H., Bearder, J.R., Down, G.H, Hutchison, M., MacMillan, J., Phinney, B.O., 1982. The biosynthesis of kaurenolide diterpenoids by *Gibberella fujikuroi*. Phytochem. 21, 1279–1287.

Bearder, J.R., MacMillan, J., Phinney, B.O., 1975. Fungal products. Part XIV. Metabolic pathways from *ent*-kaurenoic acid to the fungal gibberellins in mutant B1-41a of *Gibberella fujikuroi*. J. Chem. Soc. Perkin Trans I 721–726.

Bearder, J.R., 1983. *In vivo* diterpenoid biosynthesis in *Gibberella fujikuroi*: The pathway after *ent*-kaurene. In: Crozier, A. (Ed.), The Biochemistry and Physiology of Gibberellins. Praeger Publishers, pp. 251–387.

Cross, B.E., Galt, R.H.B., Hanson, J.R., Curtis, P.J., Grove, J.F., Morrison, A., 1963a. New metabolites of *Gibberella fujikuroi*. Part III. The isolation of fourteen new metabolites. J. Chem. Soc. 2937–2943.

Cross, B.E., Galt, R.H.B., Hanson, J.R., 1963b. New metabolites of *Gibberella fujikuroi*. Part III. The structure of 7-hydroxykaurenolide. J. Chem. Soc. 2944–2961.

Cross, B.E., Galt, R.H.B., Norton, K., 1968. The biosynthesis of gibberellins—II. Tetrahedron 24, 231–237.

Cross, B.E., Stewart, J.C., Stoddart, J.L., 1970. 6β,7β-Dihydroxykaurenoic acid: its biological activity and possible role in the biosynthesis of gibberellic acid. Phytochem. 9, 1065–1071.

Davidson, S.E., Elliot, R.C., Helliwell, C.A., Poole, A.T., Reid, J.B., 2003. The pea gene *NA* encodes *ent*-kaurenoic acid oxidase. Plant Physiol. 131, 335–344.

Edebo, L., 1983. Disintegration of cells by extrusion under pressure. In: Lafferty, R.M. (Ed.), Enzyme Technology. Springer Verlag, Berlin.

Fernández-Martín, R., Reyes, F., Domenech, C.E., Cabrera, E., Bramley, P.M., Barrero, A.F., Avalos, J., Cerdá-Olmedo, E., 1995. Gibberellin biosynthesis in *gib* mutants of *Gibberella fujikuroi*. J. Biol. Chem. 270, 14970–14974.

Gaskin, P., MacMillan, J., 1975. Polyoxygenated *ent*-kauranes and water-soluble conjugates in seeds of *Phaseolus coccineus*. Phytochem. 14, 1575–1578.

Gaskin, P., MacMillan, J., 1992. GC/MS of gibberellins and related compounds: methodology and a library of reference spectra. Cantocks enterprises, Bristol.

Geissman, T.A., Verbiscar, A.J., Phinney, B.O., Cragg, G., 1966. Studies on the biosynthesis of gibberellins from (−)-kaurenoic acid in cultures of *Gibberella fujikuroi*. Phytochem. 5, 933–947.

Graebe, J.E., Hedden, P., 1974. Biosynthesis of gibberellins in a cell-free system. In: Schreiber, K., Schütte, H.R., Sembdner, G. (Eds), Biochemistry and Chemistry of Plant Growth Regulators. Academy of Science of the German Democratic Republic, Institute of Plant Biochemistry, pp. 1–16.

Graebe, J.E., Hedden, P., Gaskin, P., MacMillan, J., 1974. Biosynthesis of gibberellins A_{12}, A_{15}, A_{24}, A_{36} and A_{37} by a cell-fee system from *Cucurbita maxima*. Phytochem. 13, 1433–1440.

Graebe, J.E., Hedden, P., MacMillan, J., 1975. The ring contraction step in gibberellin biosynthesis. J. Chem. Soc. Chem. Comun. 161–162.

Hanson, J.R., Hawker, J., White, A.F., 1972. Studies in terpenoid biosynthesis. Part IX. The sequences of oxidation on ring B in kaurene-gibberellin biosynthesis. J. Chem. Soc. Perkin Trans. 1 1892–1895.

Hedden, P., MacMillan, J., Grinsted, M.J., 1973. Fungal products. Part VIII. New kaurenolides from *Gibberella fujikuroi*. J. Chem. Soc. Perkin Trans. 1 2773–2778.

Hedden, P., Graebe, J.A., 1981. Kaurenolide biosynthesis in a cell-free system from *Cucurbita maxima* seeds. Phytochem. 20, 1011–1015.

Hedden, P., 1983. *In vitro* metabolism of gibberellins. In: Crozier, A. (Ed.), The Biochemistry and Physiology of Gibberellins. Praeger Publishers, pp. 99–149.

Hedden, P., Graebe, J.E., 1981. Kaurenolide biosynthesis in a cell-free system from *Cucurbita maxima* seeds. Phytochem. 20, 1011–1015.

Hedden, P., Phillips, A.L., Rojas, M.C., Carrera, E., Tudzynski, B., 2002. Gibberellin biosynthesis in plants and fungi: a case of convergent evolution? J. Plant Growth Regul. 20, 319–331.

Helliwell, C.A., Chandler, P.M., Poole, A., Dennis, E.S., Peacock, W.J., 2001. The CYP88A cytochrome P450, *ent*-kaurenoic acid oxidase, catalyzes three steps of gibberellin biosynthesis pathway. Proc. Natl. Acad. Sci. U.S.A. 98, 2065–2070.

Ostovic, D., Bruice, T.C., 1992. Mechanism of alkene epoxidation by iron, chromium and manganese higher valent oxo-metalloporphyrins. Acc. Chem. Res. 25, 314–320.

Rojas, M.C., Hedden, P., Gaskin, P., Tudzynski, B., 2001. The *P450-1* gene of *Gibberella fujikuroi* encodes a multifunctional enzyme in gibberellin biosynthesis. Proc. Natl. Acad. Sci. U.S.A. 98, 5828–5834.

Sono, M., Roach, M.P., Coulter, E.D., Dawson, J.H., 1996. Heme-containing oxygenases. Chem. Rev. 96, 2841–2887.

Tudzynski, B., Hölter, K., 1998. Gibberellin biosynthetic pathway in *Gibberella fujikuroi*: evidence for a gene cluster. Fungal Genet. Biol. 25, 157–170.

Urrutia, O., Hedden, P., Rojas, M.C., 2001. Monooxygenases involved in GA_{12} and GA_{14} synthesis in *Gibberella fujikuroi*. Phytochem. 56, 501–511.