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Metabolism of gibberellins and *ent*-kaurenoids in mutants of *Gibberella fujikuroi*

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Abstract

The gibberellin precursors — *ent*-kaur-16-ene (**1**), *ent*-kaur-16-en-19-ol (**2**), *ent*-kaur-16-en-19-al (**3**) and *ent*-kaur-16-en-19-oic acid (**4**) — were fed to *Gibberella fujikuroi* gibberellin-defective mutants SG121, SG136, SG138 and SG139 in order to determine at which point their metabolic pathways were blocked. Mutant SG138 was also incubated with GA_4 and GA_{14}, and with 3β-hydroxy-*ent*-kaur-16-en-19-oic acid (**10**). Results indicated that SG139 was a regulatory mutant, and the synthesis of **1** was inhibited in SG121 and SG136. SG138 was blocked in the **1**→**4** oxidation steps, as well as in the C-3 and C-13 hydroxylations and the C-20 loss. The complex phenotype showed by SG138 supports the hypothesis of a gene product involved in more than one biochemical oxidation step of the gibberellin biosynthesis. Biotransformation of GA_{14} by SG138 gave GA_{36} and GA_{37}, providing new experimental evidence about the metabolism of GA_{14} in *Gibberella*. Compound **10** was transformed to 3β,7β-dihydroxy-*ent*-kaur-16-en-19-oic acid, indicating that 3β-hydroxy group prevents enzymatic ring B contraction of *ent*-kaurenoids to gibberellins in strain SG138.

Keywords: Gibberella fujikuroi; Hyphomycetes; Mutants; Biosynthesis; Gibberellins; *Ent*-kaurenoids

1. Introduction

Gibberellins (GAs), most notably gibberellic acid (GA_3), are growth-promoting phytohormones important for agriculture and the brewing industry (Brückner, Blechschmidt, Sembdner, & Schneider, 1989). Present in trace amounts in plants, they are abundantly produced by C-group strains of the fungus *Gibberella fujikuroi*. This microorganism has been the subject of much research aimed at improving gibberellin production and establishing the biosynthetic steps in the pathway. Mutants are a useful tool both for increasing gibberellin production and for elucidating the genes and enzymes involved in their biosynthesis. In this way, mutants B1-41a (from the GF-1a wild strain (Bearder, Hedden, MacMillan, Wels, & Phinney, 1973)) and R9 (obtained from the wild-type strain N3844 (Bearder, MacMillan, & Phinney, 1973)) were employed. B1-41a does not synthesize GAs and is blocked in the oxidation step from *ent*-kaur-16-en-19-al to *ent*-kaur-16-en-19-oic acid (Bearder et al., 1973). R9 does not synthesize GA_1 or GA_3, but does produce their precursors GA_4 and GA_7, because the gibberellin C-13 hydroxylation step is blocked (Bearder et al., 1973). These mutants, especially B1-41a, have shown their utility for the study of the biosynthesis of GAs (MacMillan, 1997, and references therein). However, some hypothetical metabolic steps have not yet been experimentally proven. As a relevant example, the C-20 oxidation of the fungal gibberellin GA_{14} leading to the alcohol (lactone) GA_{37}, the aldehyde GA_{36}, and the subsequent C-20 loss to give GA_4, have not been demonstrated in *G. fujikuroi*. (MacMillan, 1997, and references therein).

Recent research into the metabolites produced by the wild-type strain IMI58289 of *G. fujikuroi* has been carried out at our laboratory (Barrero et al., 1992, 1993). Additionally, Candau, Ávalos and Cerdá-Olmedo (1991) isolated several *gib* mutants from this strain through the modified gibberellin fluorescence of their culture media (Candau et al., 1991) The chemical analysis of these mutants showed that four of them (SG121, SG136, SG138 and SG139) produced no gibberellins, fujenal, or kaurenolides, or only very small amounts, pointing to a virtually complete block in a step before *ent*-kaur-16-en-19-oic acid (Fernández-Martín et al., 1995). The aim of the present work was to make progress in the phenotype

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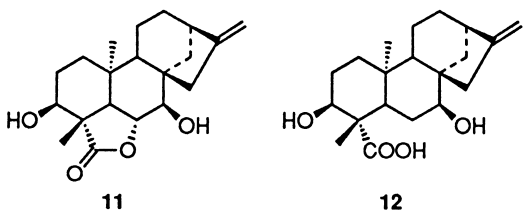
characterization of these four mutants by means of feeding experiments with the *ent*-kaurenoids **1**–**4**. The results obtained with the SG138 mutant strain indicated that it was blocked not only at the *ent*-kaur-16-ene (**1**) oxidation but also at the gibberellin C-3 hydroxylation and other biosynthetic steps. This fact prompted us to perform incubations of the SG138 mutant with the 3β-hydroxylated compounds GA_{14}, GA_4 and 3β-hydroxy-*ent*-kaur-16-en-19-oic acid. This should allow us to collate information about the fungal biosynthesis of GA_{36} and GA_{37}, and other aspects of the pathway after the gibberellin 3β-hydroxylation.

2. Results

Our aims in the feeding experiments were to differentiate between the biotransformation products and the endogenous metabolites, as well as to determine the identity of the former and their approximate proportions if possible. An accurate measurement of the amounts formed was not within the scope of our work. Hence, we followed an analytical procedure based on the extensive use of blank experiments as references for the endogenous metabolites. In all cases the mutant strain was incubated in two parallel experiments: one in a medium containing the exogenous substrate (biotransformation experiment) and the other in the same medium without exogenous substrate (blank experiment). The culture filtrates and the mycelia from both experiments were extracted, and each extract was split into neutral and acidic parts, adequately derivatized and analyzed by GC–MS. Each chromatogram from the biotransformation experiment was compared with the corresponding counterpart from the blank. In the chromatograms from the biotransformation some peaks enhanced their area with respect to those from the blank ones, and some new peaks appeared. The peak area increments and the new peaks were attributed either to biotransformation products or to the exogenous substrate remaining unchanged after incubation. The identification of the biotransformation products detected was performed on the basis of the mass spectra of their GC–MS derivatives, which matched those previously described for fujenal (dicarboxylic acid form, **5**), fujenoic acid (**6**), kaurenolide **11**, dihydroxylated acid **12** (Fig. 1), and all GAs contained in Tables 1–4 (Gaskin & MacMillan, 1991). Percentages referred to in the tables were determined as follows. The sum total of the new peak areas and the area increments of the enhanced peaks



11 **12**

Fig. 1. Chemical structures of the metabolites **11** and **12** derived from the biotransformation of **10** by SG138 mutant strain.

Table 1
Percentages of the unchanged exogenous substrates (ES) **1**–**4** and their biotransformation products after incubation with the SG121 mutant strain

	Substrates			
Products	**1**	**2**	**3**	**4**
Unchanged ES	0	0	0	0
5	17	17	19	16
6	0	3	3	1
GA_3	9	7	9	10
GA_4	7	14	10	13
GA_7	58	36	39	52
GA_9	3	2	2	3
GA_{13}	3	10	9	3
GA_{14}	0	3	2	0
GA_{16}	3	4	3	2
GA_{25}	0	4	4	0

Table 2
Percentages of the unchanged exogenous substrates (ES) **1**–**4** and their biotransformation products after incubation with the SG136 mutant strain

	Substrates			
Products	**1**	**2**	**3**	**4**
Unchanged ES	0	0	0	0
5	17	10	19	12
6	1	4	0	1
GA_1	4	6	3	5
GA_3	3	13	11	12
GA_4	52	43	35	36
GA_7	7	6	7	12
GA_{13}	14	13	21	18
GA_{14}	1	1	1	1
GA_{16}	0	1	2	2
GA_{25}	1	3	1	1

Table 3
Percentages of the unchanged exogenous substrates (ES) **1**–**4** and their biotransformation products after incubation with the SG138 mutant strain

	Substrates			
Products	**1**	**2**	**3**	**4**
Unchanged ES	100	100	100	0
5	0	0	0	2
GA_{15}	0	0	0	58
GA_{24}	0	0	0	40

Table 4
Percentages of the unchanged exogenous substrates (ES) GA_{14}, GA_4 and **10**, and their biotransformation products after incubation with the SG138 mutant strain

	Substrates		
Products	GA_{14}	GA_4	**10**
Unchanged ES	65	0	0
11	0	0	3
12	0	0	95
GA_7	0	91	0
GA_{16}	0	9	0
GA_{36}	16	0	1
GA_{37}	5	0	1
GA_{42}	14	0	0

was taken as 100% of the exogenous substrate remaining plus the biotransformation products formed after incubation. The percentage of each product was calculated directly from the area of their corresponding new peak or the area increment in the case of an enhanced peak. Obviously, these percentages are only approximate, because of different response of certain gibberellins in the GC–MS. Thus, the discussion and conclusions in this paper have been mainly based on the identity of the biotransformation products detected.

In the incubations of exogenous substrates **1**–**4** with the SG139 mutant, no biotransformation products were detected. The biotransformation products from **1**–**4** identified after incubations with the mutants SG121, SG136 and SG138, appear in Tables 1–3, respectively. The amounts of GA_3 and GA_7 include those of their respective artifacts, GA_3^*, *iso*-GA_3, GA_7^* and *iso*-GA_7 (Fernández-Martín et al., 1995).

GA_{15} and GA_{24}, two C_{20} 3,13-nonhydroxylated gibberellins, were the only gibberellins generated by the SG138 strain from **4** Table 3. In order to gather more information on such an unusual mutant, we checked its ability to transform the 3β-hydroxygibberellins GA_{14} (C_{20}) and GA_4 (C_{19}) Table 4. This mutant was also a useful tool to obtain information about the effect of a 3β-hydroxyl group on the activity of the enzymatic system involved in the ring B contraction from kaurenoid to gibberellin skeleton. Therefore, we synthesized the previously nondescribed 3β-hydroxy-*ent*-kaur-16-en-19-oic acid (**10**) (see below) and performed its incubation with the SG138 mutant. Results are shown in Table 4. It is noteworthy that all GAs referred in Tabs. 1–4 were always found in the culture filtrate extracts and were never detected in the mycelium extracts.

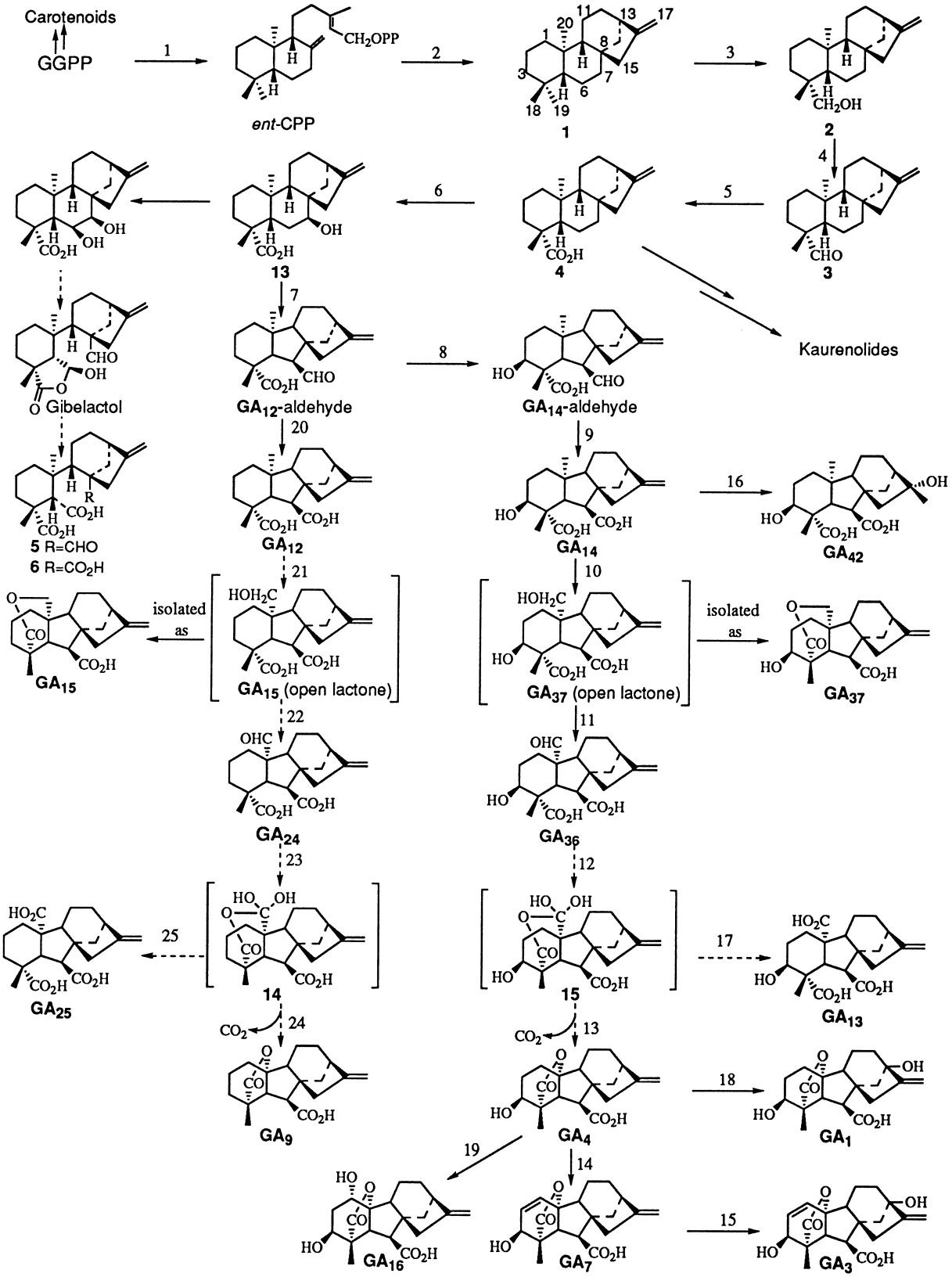
Acid **12**, the main product from the biotransformation of **10**, could be isolated as its methyl ester. Analysis of the ^1H, ^{13}C, and 2-D NMR spectra of this ester ratified the proposed structure **12**. Moreover, in the ^1H NMR spectrum, the signal of H-7 (δ 3.62, dd, $J=3.4$, 2.2 Hz) showed coupling constant values confirming its α-equatorial location. *Ent*-kaurenoids, GAs and fujenal related compounds found in wild-type strain IMI58289 (Barrero et al., 1992; Fernández-Martín et al., 1995) as well as in the mutant strains SG121, SG136 and SG138 are depicted in Scheme 1. This scheme is adapted from the metabolic pathway of GAs and *ent*-kaurenoids proposed for ACC917 wild-type strain, and R-9 and B1-41a mutants (Bearder, 1983). The changes in the scheme are explained in Section 3. In IMI58289, the main pathway goes from geranylgeranyl pyrophosphate (GGPP) to GA_3 (steps 1–15, Scheme. 1), but a lot of carbon atom flow is diverted to the synthesis of gibelactol, fujenal (**5**) and fujenoic acid (**6**).

997. *Synthesis of 3β-hydroxy*-ent-*kaur-16-en-19-oic acid (**10**)*

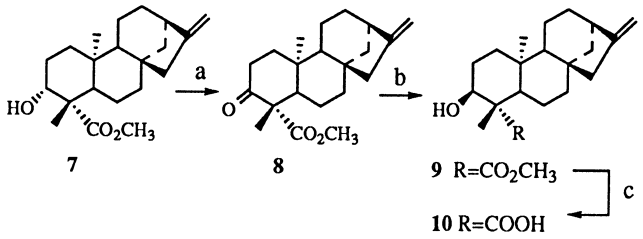
Synthesis of **10** was performed starting from methyl ester **7** (Scheme 2), isolated from the esterified acidic fraction of an extract from the plant *Odontites longiflora* (Barrero, Riu, Ramírez, & Altarejos, 1988). Oxidation of **7** gave the ketone **8**, which was stereoselectively reduced to a mixture of methyl 3β-hydroxy-*ent*-kaur-16-en-19-oate (**9**, 92%) and methyl 3α-hydroxy-*ent*-kaur-16-en-19-oate (**7**, 8%). In the ^1H NMR spectrum of **7**, H-3 showed chemical shift (3.05 ppm) and coupling constant values ($J_{3,2\alpha}=12$, $J_{3,2\beta}=4$ Hz) indicating its β-axial position, whereas in **9** H-3 appeared at δ 4.13 (t, $J=3$ Hz) showing its α-equatorial location. Ester **9** was recovered unchanged after boiling in hydroalcoholic KOH. Finally, the acid **10** was obtained by treating **9** with sodium 1-propanethiolate.

3. Discussion

In unsupplemented cultures, strains SG139 and SG121 produced very little *ent*-kaur-16-ene (**1**) and no detectable gibberellins, fujenal or kaurenolides; but their carotenoid biosynthesis was not affected, indicating the ability to produce GGPP (Fernández-Martín et al., 1995). This phenotype was explained on the basis of either a defective kaurene synthetase or a regulatory mutation (Fernández-Martín et al., 1995). The failure of SG139 to metabolize the *ent*-kaurenoids **1**–**4** supported it being a regulatory mutant, unable to carry out at least the reactions up to 7β-hydroxy-*ent*-kaur-16-en-19-oic acid in the pathway. On the other hand, SG121 completely biotransformed the four *ent*-kaurenoids into gibberellins Table 1, indicating that it is defective in kaurene biosynthesis, that is, either in the *ent*-copalyl pyrophosphate (*ent*-CPP) synthase (Scheme. 1, step 1), in the *ent*-kaur-16-ene synthase (Scheme. 1, step 2) or in both activities. The two enzymes which catalyze the cyclization of GGPP to *ent*-kaur-16-ene (**1**), via *ent*-CCP, are separate gene products in plants



Scheme 1. Gibberellin biosynthesis pathway in the wild-type strain IMI58289 and SG121, SG136 and SG138 mutants of *Gibberella fujikuroi*. Dotted arrows are experimentally not proved steps. In the mutant strains the following steps were blocked: in SG121, 1 or 2; in SG136 in vivo, 1 or 2; in SG138, 3, 4, 5, 8, 12, 15, 18 and 23.



Scheme 2. Synthesis of 3β-hydroxy-*ent*-kaur-16-en-19-oic acid. (a) Jones' reagent (b) $(iPrO)_3Al/iPrOH$ (c) C_3H_7SNa.

and *Escherichia coli* (MacMillan, 1997). However, early attempts to purify these enzymes from *G. fujikuroi* failed to dissociate both activities (Fall & West, 1971). In the biotransformations with mutant SG121, no GA_1 was observed.

In unsupplemented cultures, strain SG136 synthesized the same carotenoids as the wild type, but produced only very small amounts of gibberellins and fujenal late in the growth cycle; in contrast, mycelial extracts synthesized *ent*-kaur-16-ene (**1**) in vitro (Fernández-Martín et al., 1995). The complete biotransformation of **1** into **5**, **6** and GAs Table 2 confirmed that this mutant lacks the biosynthesis of **1** from GGPP in vivo.

The results obtained with strain SG138 were particularly interesting. In unsupplemented cultures, it accumulated *ent*-kaur-16-ene (**1**) in vitro and in vivo, and only small amounts of GA_{15} and GA_{24} (Fernández-Martín et al., 1995). These facts were justified by a defect in the oxidation of **1** to **4** and additional defects in the hydroxylation at carbon 3 and in the loss of carbon 20 of GAs. In contrast with SG121 and SG136, strain SG138 was unable to metabolize **2** and **3** Table 3, confirming that it was blocked not only at step 3 in Scheme. 1, but also at steps 4 and 5. Moreover, its biotransformation of **4** yielded an unusually simple mixture of GA_{15} and GA_{24}, besides a small amount of **5** Table 3. No formation of GA_{25}, GA_9 or 3-hydroxylated GAs was observed. Absence of hydroxylated GAs confirmed an additional block at the gibberellin C-3 hydroxylation step (from GA_{12}-aldehyde to GA_{14}-aldehyde). Obviously, the minor 3-hydroxylation step from GA_{12} to GA_{14}, which operates in mutant B1-41a (MacMillan, 1997, and references therein) did not occur in SG138. GA_{25} is not a precursor of GA_9 in mutant B1-41a (Bearder et al., 1973). Assuming that this observation is also true for SG138, lack of both GA_{25} and GA_9 in this mutant can be explained by a simple defective oxidation step (Scheme. 1, step 23) only if a common intermediate between GA_{24} and both GA_{25} and GA_9 is accepted. This intermediate could be the anhydride hydrate **14**, which was postulated by MacMillan (1997) (and references therein) in the mechanistic justification for the formation of GA_{25} and GA_9 from the GA_{24} lactol form.

The biotransformation of GA_{14} by strain SG138 Table 4 is the first experimental evidence for the fungal biogenesis of GA_{37}. Thirty years ago, Cross, Norton and Stewart (1968) proposed the general pathway of GA_3 biosynthesis in *Gibberella*. They also established that GA_{13} is not a precursor of GA_3, but that it is formed by the irreversible oxidation of a precursor, possibly GA_{14}. Since then, metabolic transformation of GA_{14} (a C_{20}-gibberellin) into GA_4 (the first C_{19}-gibberellin before GA_3) has been a subject of controversy. The sequence depicted in Scheme. 1 ($GA_{14}\rightarrow GA_{37}$ open lactone$\rightarrow$ $GA_{36}\rightarrow$**15**$\rightarrow GA_4$) is adapted from the general pathway from GA_{12}-aldehyde to C_{19}–GAs proposed by MacMillan (1997). This scheme agrees with the findings of Cross et al. (1968) and is supported by the results summarized in Table 4. A block at step 12 of the scheme, which leads to a hypothetical intermediate such as **15** (the 3-hydroxylated counterpart of **14**), would explain the lack of GA_{13} and all of C_{19}–GAs (downstream biotransformation products of GA_{14}).

These observations suggest a close relationship between the enzymatic systems catalyzing the metabolism of GA_{12} and GA_{14} in *G. fujikuroi*. It is generally accepted that plant soluble oxidases, requiring Fe^{2+}, 2-oxoglutarate and ascorbate as cofactors, catalyze the steps from GA_{12} to GA_9 and GA_{14} to GA_4 (MacMillan, 1997, and references therein). It is also assumed that the GA 20-oxidases of plants are multifunctional in that they catalyze some or all of the steps in the sequential oxidation of GA_{12} at C-20 to the alcohol GA_{15}, the aldehyde GA_{24}, the acid GA_{25} and the C_{19}–gibberellin GA_9 (MacMillan, 1997, and references therein). In *G. fujikuroi*, there are no data available about the enzymology of these metabolic steps. However, biotransformations of **4** Table 3 and GA_{14} Table 4 by strain SG138 suggest differences between the gene product catalyzing the C-20 oxidations $GA_{12/14}\rightarrow GA_{15/37}\rightarrow GA_{24/36}$ and those involved in the subsequent transformations of the aldehydes GA_{24} and GA_{36}.

Biotransformation of GA_4 Table 4 by strain SG138 mainly produced GA_7, whereas GA_1 and GA_3 were not detected. This observation points to another blocked biochemical reaction, the C-13 hydroxylation of GA_4 and GA_7 (Scheme. 1, steps 18 and 15). The number of defective metabolic steps in mutant SG138 is unusually high. It has been proposed that a gene product might be involved in more than one different biochemical oxidation in the GAs pathway (Fernández-Martín et al., 1995). The complex phenotype exhibited by SG138 supports this hypothesis. Alternative explanations, such as more than one gene affected, or a regulatory mutation affecting only one set of reactions, seem very unlikely.

The partial substrate specificity of several fungal enzymes involved in the gibberellin biosynthesis is known (MacMillan, 1997, and references therein). Thereby, a wide range of 'unnatural substrates' may be metabolized by *G. fujikuroi* giving 'unnatural' fungal GAs (MacMillan, 1997, and references therein). This partial speci-

ficity for the substrate may have general validity not only for the terpenoids, but in other cases of secondary metabolism (Cerdá-Olmedo, 1994). However, some fungal enzymes exhibit strict chemical requirements for a given site of the substrate molecule. In this way, 3α-hydroxy-*ent*-kaur-16-ene was not metabolized by the wild-type strain IMI58289 of *G. fujikuroi*, because the 3α-OH group inhibits hydroxylation at C-19 (Scheme .1, step 3) (Fraga, González, Hanson, & Hernández, 1981). In the B1-41a mutant and in its parent strain GF-1a, a partial inhibition of the enzymatic activity responsible for the ring B contraction of *ent*-kaurenoids to GAs, was observed (Lunnon, MacMillan, & Phinney, 1977). In the SG138 mutant (derived from the wild-type strain IMI58289) the inhibition of ring B contraction by the 3β-OH group was stronger than in B1-41a. Therefore, the biotransformation of 3β-hydroxy-*ent*-kaur-16-ene-19-oic acid by SG138 produced only small amounts of GAs, with 3β,7β-dihydroxy-*ent*-kaur-16-ene-19-oic acid (**12**) being the main product Table 4. Inhibition of ring B contraction of **12** was due to the lack of enzymatic molecular recognition, and not to transport difficulties, because **12** was generated in situ by the fungus itself. This phenomenon forces the 3-hydroxylation step to occur after the ring contraction in the metabolic pathway. In this way, the specificity for a concrete molecular element, showed by some enzymes, may serve as a selective filter favoring a specific reaction sequence in the GA biosynthesis.

3. Experimental

3.1. General methods

Optical rotations were determined on a Perkin-Elmer 141 polarimeter. Melting points were taken using a Reichert apparatus and are uncorrected. NMR spectra were recorded on a Bruker AMX 300 and a Bruker ARX 400 spectrometers using tetramethyl silane (TMS) as an internal standard. Chemical shifts are reported in parts per million (δ) relative to TMS and coupling constants (J) are in Hertz. Carbon substitution degrees in ^{13}C NMR spectra were established by DEPT multipulse sequence. COSY, HMQC and HMBC experiments were carried out using standard Bruker software. HRMS were registered on an Autospec-Q VG-Analytical (FISONS) mass spectrometer. Chromatographic separations were carried out in a conventional silica gel (Merck 60, 230–400 mesh) column or by flash chromatography. Analytical and preparative thin layer chromatography (TLC) was performed on silica gel 60 F_{254} Merck plates.

3.2. Mutant strains

The four *gib* mutants were derived from the wild type strain IMI58289 of *G. fujikuroi*, ssp. *fujikuroi*, after exposure of its spores to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Candau et al., 1991).

3.3. General biotransformation procedure

The growing medium was ICI medium (Geissman, Verbiscar, Phinney, & Cragg, 1966) with 2.4 g/l of NH_4NO_3. The sterilized growing medium (50 ml, in 500-ml Erlenmeyer flasks) was inoculated with spores obtained from cultures on sporulation agar (Cerdá-Olmedo, Fernández-Martín, & Avalos, 1994) and incubated (orbital shaker, 200 rpm, 30°C, darkness) for 4 days. Fresh ICI medium with 2.4 g/l of NH_4NO_3 (50 ml, in 500-ml Erlenmeyer flasks) was inoculated with 2 ml of the culture described above and incubated for another 4 days. The mycelium was then separated by filtration and resuspended in ICI medium without NH_4NO_3 (50 ml, in 500-ml Erlenmeyer flasks). An ethanolic or acetonic solution of the substrate (3 mg/100 μl) was added to each flask of the above fungal suspension and incubation was continued for another 72 h.

3.4. Extraction and GC–MS analysis procedures

The culture media were separated from the mycelia by filtration and brought to pH 8 with NaOH. Alkaline solution was extracted three times with EtOAc, brought to pH 2 with HCl, and extracted three more times with EtOAc. The dried acid extracts were treated with diazomethane mixed with pyridine and *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) (10 μl of both per mg of extract), and heated at 110°C for 20 min, to form methyl trimethylsilyl derivates for GC–MS analysis. The alkaline extracts were derivatized with BSTFA/pyridine as above. Mycelia were washed, dried, ground in a mortar and extracted with EtOAc for 24 h. The extracts (containing abundant triglycerides) were saponified with 2 N KOH in methanol (1 ml per 100 mg of extract) for 12 h, diluted with water and extracted with EtOAc. Aqueous layer was brought to pH 2 with HCl and extracted with EtOAc. Both extracts (alkaline and acid) were analyzed as the culture medium extracts.

GC–MS analyses were performed with a Hewlett Packard 5890A gas chromatograph coupled to a Hewlett Packard 5972 mass spectrometer using an ionizing voltage of 70 eV (EIMS). Gas chromatography was carried out on a capillary column HP-1 of cross-linked methylsilicone gum, 25 m × 0.2 mm × 0.33 μm film thickness; programmed temperature increase from 120 to 220°C at 5°C/min, from 220 to 280°C at 3°C/min and 280°C for 10 min; injector temperature 260°C; carrier gas He at 0.6 ml/min.

Biotransformation products included in Tables 1–4 were identified by comparison of mass spectra with those previously described (Gaskin & MacMillan, 1991) and by

using our own standards (Barrero et al., 1992; Fernández-Martín et al., 1995).

3.4.1. Ent-*kaur-16-ene* (***1***)

It was obtained from **3** as previously reported (Barrero et al., 1988).

3.4.2. Ent-*kaur-16-en-19-ol* (***2***)

It was isolated from the plant *O. longiflora* as previously described (Barrero et al., 1988).

3.4.3. Ent-*kaur-16-en-19-al* (***3***)

It was obtained from **2** as previously reported (Barrero et al., 1988).

3.4.4. Ent-*kaur-16-en-19-oic acid* (***4***)

It was generously donated by Professor M. Grande (Universidad de Salamanca, Salamanca, Spain).

3.4.5. Methyl 3α-hydroxy-ent-*kaur-16-en-19-oate* (*7*)

It was isolated from *O. longiflora* as previously reported (Barrero et al., 1988).

3.4.6. Methyl 3-oxo-ent-*kaur-16-en-19-oate* (***8***)

Jones' reagent (0.3 ml, 0.8 mmol) was slowly added to a stirred solution of **7** (225 mg, 0.68 mmol) in acetone (5.6 ml) and *t*-BuOMe (0.5 ml) at 0°C. The mixture was stirred for 30 min. H_2O (18 ml) was added and the mixture was extracted with *t*-BuOMe. The organic layer was washed with H_2O, dried over anh. Na_2SO_4 and the solvent removed. Ketone **8** (224 mg) was obtained. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.12 (3H, s, H-20), 1.35 (3H, s, H-18), 2.07 (2H, br s, H-15), 2.16 (1H, ddd, $J_{1\alpha,2\beta}=2$, $J_{1\alpha,2\alpha}=7$, $J_{1\alpha,1\beta}=13$ Hz, H-1α), 2.37 (1H, ddd, $J_{1\alpha,2\beta}=2$, $J_{2\beta,1\beta}=5$, $J_{2\beta,2\alpha}=15$ Hz, H-2*β*), 2.67 (1H, m, H-13), 2.94 (1H, ddd, $J_{1\alpha,2\alpha}=7$, $J_{2\beta,2\alpha}=15$, $J_{2\alpha,1\beta}=15$ Hz, H-2α), 3.67 (3H, s, OCH_3), 4.74 (1H, br s, H-17a), 4.81 (1H, m, H-17b); EIMS *m*/*z* (rel. int.) 330 $[M]^+$ (44), 312 (8), 287 (58), 255 (68), 227 (55), 187 (52), 105 (63), 91 (100).

3.4.7. Methyl 3β-hydroxy-ent-*kaur-16-en-19-oate* (***9***)

Compound **8** (100 mg, 0.3 mmol) was added to a solution of aluminium isopropoxide (5 g, 24 mmol) in propan-2-ol (59 ml). The mixture was stirred and heated for 1h and 15 min, distilling off 8 ml of propan-2-ol. H_2O (80 ml) and EtOAc (50 ml) were added and the mixture was filtered. Filtrate was dried and more water was added, then extracted with EtOAc. The organic layer was washed with H_2O, dried over anh. Na_2SO_4 and the solvent removed. The flash chromatography (hexane:*t*-BuOMe 7:3) yielded **9** (84 mg), $[\alpha]_D$ −65.7 (*c* 0.96, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.86 (3H, s, H-20), 1.29 (3H, s, H-18), 2.07 (2H, m, H-15), 2.67 (1H, br s, H-13), 3.68 (3H, s, OCH_3), 4.13 (1H, t, $J=3$Hz, H-3), 4.76 (1H, br s, H-17a), 4.82 (1H, m, H-17b); EIMS (trimethyl silyl ether (TMSi) derivative) *m*/*z* (rel. int.) 404 $[M]^+$ (2), 389 (8), 287 (58), 314 (26), 275 (89), 187 (100), 129 (23). 7 mg of **7** were also isolated.

3.4.8. 3β-hydroxy-ent-*kaur-16-en-19-oic acid* (***10***)

Compound **9** (100 mg, 0.30 mmol) was dissolved in 6 ml of a solution 0.57 M of C_3H_7SNa in HMPA in anhydrous conditions under an inert atmosphere. The mixture was stirred for 8 h at room temperature. Then, it was added to H_2O and it was brought to pH 2. Water solution was extracted with *t*-BuOMe. The organic layer was dried over anh. Na_2SO_4 and the solvent removed. Preparative TLC (AcOEt:hexane:AcOH 40:60:1) of the residue yielded 46 mg of **10** ($R_f=0.6$). M.p. 212–214°C; $[\alpha]_D$ −47.4 (*c* 1.03, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.95 (3H, s, H-20), 1.33 (3H, s, H-18), 2.63 (1H, br s, H-13), 4.08 (1H, br s, H-3), 4.73 (1H, br s, H-17a), 4.79 (1H, br s, H-17b); ^{13}C NMR (MeOH-d_4, 100 MHz) δ 15.3 (q, C-20), 18.4 (t, C-11), 24.3 (q, C-18), 26.3 (t, C-2), 21.4 (t, C-6), 33.1 (t, C-12), 33.9 (t, C-1), 39.4 (t, C-14), 39.7 (s, C-10), 41.1 (t, C-7), 48.9 (d, C-5), 44.2 (d, C-13), 48.9 (t, C-15), 47.5 (s, C-4), 43.8 (s, C-8), 54.8 (d, C-9), 70.9 (d, C-3), 103.1 (t, C-17), 155.8 (t, C-16), 182.2 (s, C-19); EIMS (TMSi derivative) *m*/*z* (rel. int.) 462 $[M]^+$ (3), 447 (11), 372 (12), 357 (17), 333 (67), 187 (76), 129 (13), 73 (100).

3.4.9. GA_4

It was isolated from a culture of the wild-type strain IMI58289 of *G. fujikuroi*.

3.4.10. GA_{14}

It was generously donated by Professor B.M. Fraga (Instituto de Productos Naturales y Agrobiología, La Laguna, Tenerife, Canary Islands, Spain).

3.4.11. Methyl 3β,7β-dihydroxy-ent-*kaur-16-en-19-oate* (***12*** *methyl ester*)

Acidic fraction (70 mg) of the culture filtrate from the biotransformation of **10** by SG138 was treated with diazomethane and subsequently chromatographed over a silica gel column eluting with a hexane:*t*-BuOMe gradient. Methyl ester of **12** (12 mg) (hexane:*t*-BuOMe 40:60) was isolated. ^{1}H NMR ($CDCl_3$, 400MHz) δ 0.84 (3H, s, H-20), 1.28 (3H, s, H-18), 2.68 (1H, br s, H-13), 3.62 (1H, dd, $J=2.2$, $J=3.4$ Hz, H-7), 4.12 (1H, t, $J=2.9$ Hz, H-3), 4.80 (1H, br s, H-17a), 4.82 (1H, br s, H-17b); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 15.0 (q, C-20), 18.0 (t, C-11), 24.0 (q, C-18), 26.3 (t, C-2), 29.8 (t, C-6), 33.6 (t, C-12), 33.9 (t, C-1), 38.7 (t, C-14), 39.0 (s, C-10), 39.6 (d, C-5), 43.7 (d, C-13), 45.3 (t, C-15), 47.2 (s, C-4), 48.3 (s, C-8), 49.0 (d, C-9), 51.3 (q, C-21), 71.1 (d, C-3), 77.1 (d, C-7), 103.8 (t, C-17), 154.9 (t, C-16), 177.8 (s, C-19). The assignments of the ^{1}H and ^{13}C NMR spectra were deduced by trivial analysis of the COSY, HMQC and HMBC spectra. HREIMS *m*/*z* 348. 2295 (calcd for $C_{21}H_{32}O_4$ 348.2301); EIMS (TMSi derivative) *m*/*z* (rel.

int) 492 $[M]^+$ (3), 477 (7), 402 (11), 363 (16), 273 (100), 185 (89), 129 (25).

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