

Microbial Transformation of *ent*-Kaurenoic Acid and Its 15-Hydroxy Derivatives by the SG138 Mutant of *Gibberella fujikuroi*

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Feeding experiments with *ent*-kaurenoic acid (**4**), 15 α -hydroxy-*ent*-kaurenoic acid (**5**), 15 β -hydroxy-*ent*-kaurenoic acid (**6**), and mixtures of **4** plus **5** and **4** plus **6** were conducted using the SG138 mutant of *Gibberella fujikuroi*, to gain information about the phenotype of this unique strain. The biotransformation of **5** gave 7 β ,15 α -dihydroxykaurenolide (**9**) and 7 β ,15 α -dihydroxy-*ent*-kaurenoic acid (**13**). The incubation of **6** produced 7 β ,15 β -dihydroxy-*ent*-kaurenoic acid (**7**) and 7 β ,15 β -dihydroxykaurenolide (**10**). No 15-hydroxylated gibberellins were detected in any of these experiments. The results indicated that a hydroxy group at C-15 does not inhibit 7 β -hydroxylase activity but in the SG138 strain obstructs the enzymatic ring-B contraction of *ent*-kaurenoids to gibberellins. Exogenous **4** stimulated both the excretion of *ent*-kaurene and the fungal metabolism of **5** and **6**.

Gibberellins (GAs) are terpenoid phytohormones which play an important role in the regulation of plant growth and development. Apart from plants some fungi also produce these terpenoids, particularly *Gibberella fujikuroi* (Hypocreale). GAs have many applications in agriculture and the brewing industry.¹ Therefore, their abundant production by some strains of *G. fujikuroi* has led to the study of their biosynthesis by this fungus in considerable detail over the last three decades.² Some aspects of the biogenesis of the gibberellins via *ent*-kaurene (**1**), *ent*-kaurenol (**2**), *ent*-kaurenal (**3**), and *ent*-kaurenoic acid (**4**) are fully described,² but many of the biochemical and physiological aspects of fungal gibberellin biosynthesis are still poorly understood. As part of our research into the biotechnology of *G. fujikuroi* we have investigated the metabolites produced by the GA-producer IMI58289 strain.^{3,4} Subsequently, the kaurenoids and GAs synthesized by several *gib*-mutants developed from the IMI58289 wild-type strain were also analyzed.⁵ The results of these analyses and preliminary feeding experiments⁶ suggested to us that one of these mutants (SG138) was blocked in the oxidative transformations from *ent*-kaurene (**1**) to *ent*-kaurenoic acid (**4**), as well as in 3 β -hydroxylation, 13 β -hydroxylation, and the loss of C-20 by the GAs. These unusual features prompted us to make further feeding experiments with strain SG138 to gain more information about its unusual phenotype. We describe here the results of incubations with *ent*-kaurenoic acid (**4**), the “unnatural” substrates 15 α -hydroxy-*ent*-kaurenoic acid (**5**) and 15 β -hydroxy-*ent*-kaurenoic acid (**6**), and mixtures of **4** plus **5** and **4** plus **6**. Throughout these experiments it was observed that *ent*-kaurenoic acid (**4**) substantially stimulated the metabolism and/or excretion of **1**, **5**, and **6**.

The results of incubations using the SG138 mutant of *G. fujikuroi*, both with and without *ent*-kaurenoic acid (**4**), are summarized in Table 1. All compounds included in this table were identified by means of GC–MS. Several substances could be isolated, and their structures were confirmed by NMR techniques (see the Experimental Section

for more details). A number of blank experiments (with no exogenous substrate) were made and always gave similar results; their arithmetical means are included in Table 1. In the usual culture conditions for GA production, minimal medium with no nitrogen source (blank, Table 1), the mutant SG138 accumulated a high proportion of *ent*-kaurene (**1**) in the mycelium. The low quantities of fujenoic acid (**11**), GA₁₅ (**15**), and GA₂₄ (**16**) found in the culture medium may be ascribed to the leaky blockage in the metabolic steps from **1** to **4** of this mutant. When the fungus was fed with *ent*-kaurenoic acid (**4**), this compound was taken up into the mycelium, and the proportions of **11**, **15**, and **16** excreted were much higher. Kaurenolide (**8**) was also formed, but neither the C₁₉ gibberellins nor the C-3 or C-13 hydroxylated GAs were detected, confirming the multiple metabolic blockage of the SG138 mutant. Moreover, *ent*-kaurenoic acid (**4**) stimulated the excretion of *ent*-kaurene (**1**) into the medium. This observation prompted us to add *ent*-kaurenoic acid (**4**) to the incubations in combination with other kaurenoids in order to obtain information about its effect on the metabolism and excretion of these compounds.

The results of the feeding experiments with grandifloric acid (**5**) and with a mixture of **5** plus **4** are included in Table 1. When the fungus was fed with **5** in the absence of exogenous **4**, low proportions of the dihydroxylated metabolites **9** and **13** were synthesized by the mutant. The previously undescribed acid **13** was tentatively identified (GC–MS analysis) on the basis of the mass spectrum of its methyl ester bis-trimethylsilyl ether, closely related to that reported for the corresponding derivative of 7 β ,15 β -dihydroxy-*ent*-kaurenoic acid.⁷ When *G. fujikuroi* mutant SG138 was fed with **5** in the presence of exogenous **4**, enhanced amounts of **9** and **13** were obtained, suggesting that *ent*-kaurenoic acid stimulated the metabolism of **5**. Additionally, acid **13** could be isolated as its methyl ester **13a** (the culture broth extract was methylated before chromatography). Its HRMS showed a C₂₁H₃₂O₄ molecular formula. In the ¹H NMR spectrum several signals (H-13, H-17a, H-17b, H-18, and H-20) closely related to those of **5** were observed (see Experimental Section). Moreover, apart from the signal of a methoxy group (3.63 ppm), those of two hydroxylated methines appeared at 4.09 and 3.94

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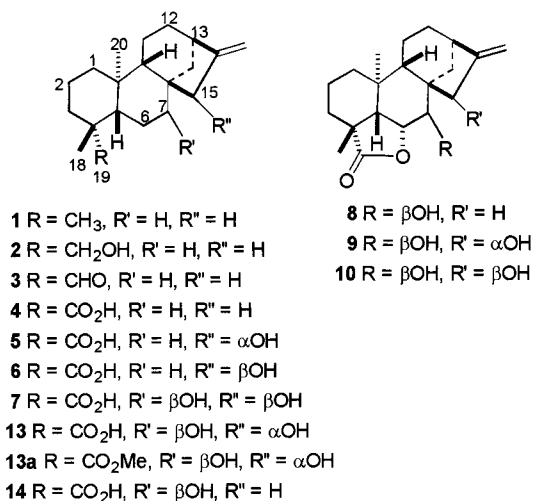
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Table 1. Kaurenoids (**1–11**, **13**) and GAs (**15**, **16**) Found after the Feeding Experiments^a

	blank		4 ^b		5 ^b		4 + 5 ^b		6 ^b		4 + 6 ^b	
	M ^c	B ^d	M ^c	B ^d	M ^c	B ^d	M ^c	B ^d	M ^c	B ^d	M ^c	B ^d
1	46.4	3.8	8.9	62.6	9.3	trace	45.6	19.7	30.0	9.2	9.4	12.0
2				0.9						trace		
3				1.2				1.1		0.2		
4			10.2	0.9	trace	trace	70.0	3.5		trace	24.0	2.0
5					27.7	4.0	trace	3.5				
6									98.7	4.4	0.7	1.9
7										4.5		52.0
8				5.5								
9						3.7		26.8				
10										0.7		6.7
11		1.9		7.3		trace						
13					6.7	1.8		48.0				
15		1.3		20.6		trace		3.3		trace		1.7
16		3.9		18.0		trace		7.9		trace		1.5

^a Proportions are in mg/L of culture broth. ^b Exogenous substrate. ^c M: Products found in the mycelium. ^d B: Products found in the culture broth.

Chart 1. Chemical Structures of Compounds **1–16**

ppm. The broad singlet at 4.09 ppm was attributed to H-15, which is on an allylic position, while the signal at 3.94 ppm (t, $J = 3$ Hz) was assigned to H-7. Its multiplicity and coupling constant indicated that H-7 was in an α -equatorial position, and thus the β -axial arrangement of the C-7 hydroxy group was established. The ¹³C NMR spectrum confirmed the structure methyl 7 β ,15 α -dihydroxy-*ent*-kaur-16-en-19-oate (**13a**). Biotransformation products from **5** with a GA skeleton were not detected in any of these experiments. These results tend to confirm that a 15 α -hydroxyl group does not inhibit *ent*-kaur-16-en-19-oate hydroxylase activity, as previously suggested by Fraga et al.⁸ On the other hand, the lack of 15-oxygenated GAs indicates that a 15 α -hydroxy group obstructs kaurenoid ring B contraction in the *G. fujikuroi* SG138 mutant. The notice-

able substrate specificity of the enzymatic system responsible for the kaurenoid ring B contraction by the SG138 mutant was already observed when feeding experiments with 3 β -hydroxy-*ent*-kaur-16-en-19-oic acid were performed in our laboratory.⁶ This specificity can be attributed to a lack of enzymatic recognition and not to transport difficulties, because **13** was synthesized in situ by the fungus itself. Another explanation, based on an enzyme deactivation derived from the mutagenesis process, seems less probable if the transformation of **4** into GA₁₅ (**15**) and GA₂₄ (**16**) carried out by the mutant (Table 1) is borne in mind.

The results of incubations with 15 β -hydroxy-*ent*-kaur-16-en-19-oic acid (**6**), and with **6** plus **4**, are summarized in Table 1. With no exogenous **4**, incubation with **6** gave low proportions of the dihydroxylated metabolites **7** and **10**. 15 β -Hydroxy derivatives with a GA skeleton were not detected, confirming that a 15-hydroxy group inhibits the kaurenoid ring B contraction in the *G. fujikuroi* mutant SG138. Subsequently, when SG138 was fed with **6** plus **4**, the peaks derived from **7** in the GC-MS chromatogram of the culture broth rose sharply, confirming the stimulating effect of **4** on the 7 β -hydroxylation of the "unnatural" 15-hydroxy-*ent*-kaur-16-en-19-oic acids. This chromatogram also showed a considerable increase in the peak corresponding to the kaurenolide **10**. It is known that 7 β -hydroxy-*ent*-kaur-16-en-19-oic acid (**8**) is biosynthesized from *ent*-kaur-16-en-19-oic acid (**4**) via *ent*-kaur-6,16-dienoic acid.⁹ Thus, the enhanced proportions of kaurenolides **9** and **10** (Table 1) suggest that exogenous **4** may also stimulate the 6,7-dehydrogenation processes of **5** and **6**. Finally, it is interesting to note that, while diol **7** was found (besides low proportions of 15 β -hydroxy gibberellins) in previously reported biotransformations of **6**,^{10,11} diol **13** was not detected in previous incubations of **5** with the ACC917 wild-type strain⁸ and with the mutant B1-41a¹² of *G. fujikuroi*.

In this work and in a previous publication⁶ we have seen that the *G. fujikuroi* mutant SG138 is very capable of taking kaurenoids from the culture broth, transforming them, and returning the transformed products to the culture medium. This capacity makes the SG138 strain a valuable biological tool for feeding experiments. This kind of experiment with "unnatural" kaurenoid and gibberellin analogues has proved to be a useful device for studying the substrate specificity of *G. fujikuroi* enzymes.^{2,13} Most of these experiments were made under conditions lacking *ent*-kaur-16-en-19-oic acid biogenesis, either with a specific mutant (such as B1-41a) or a wild-type strain in the presence of a specific inhibitor (such as AMO-1618). In many cases the quantities of biotransformed products obtained were very

low, allowing only a tentative identification on the basis of GC–MS analysis. Our findings concerning the stimulating effect of **4** could be useful in future feeding experiments of the SG138 mutant with “unnatural” substrates, allowing an enhanced production of biotransformation compounds.

Experimental Section

General Experimental Procedures. These procedures were described previously.¹⁴

Fungal Material. The development of the *G. fujikuroi* SG138 mutant from the IMI58289 wild-type strain has been reported elsewhere.^{5,15} The fungus was stored on Sabouraud agar slants at 4 °C.

ent-Kaurenoic Acid (4) and Its 15-Hydroxy Derivatives (5 and 6). *ent*-Kaur-16-en-19-oic acid (**4**) was generously donated to us by Prof. M. Grande, Department of Organic Chemistry, University of Salamanca, Spain. Its spectroscopic properties, including optical rotation, matched those reported.^{16–18} 15 α -Hydroxy-*ent*-kaur-16-en-19-oic acid (**5**) was isolated from *Helichrysum foetidum*,¹⁹ with $[\alpha]_D^{25}$ –119.8° (c 1.32, CHCl₃); its ¹H and ¹³C NMR spectra matched those described elsewhere.^{17,20} 15 β -Hydroxy-*ent*-kaur-16-en-19-oic acid (**6**) was obtained in the following manner. Methyl 15 β -hydroxy-*ent*-kaur-16-en-19-oate (500 mg), donated to us by Prof. M. Grande, was treated with sodium propanethiolate (1.44 g) in DMF (27 mL). The mixture was stirred for 45 h at 50 °C under an inert atmosphere. Water (50 mL) and 2 N HCl were added to attain pH 2. The mixture was then extracted with *t*-BuOMe, with the organic layer dried over anhydrous Na₂SO₄ and the solvent removed. Flash chromatography²¹ (hexane/*t*-BuOMe, 7:3) of the residue yielded 323 mg of **6** in the form of white needles, mp 211–212 °C, $[\alpha]_D^{25}$ –89° (c 0.77, CHCl₃) (lit.²² mp 204–206 °C, $[\alpha]_D^{25}$ –95°). Its ¹H and ¹³C NMR spectra matched those reported elsewhere.^{17,22}

Incubation Procedure. Fragments of mycelium taken from the stored agar slants were transferred to Petri dishes containing sporulation agar²³ and incubated at 28 °C for 6 days. Suspensions (1 mL) of mycelial fragments and spores, obtained by washing the surface of each agar plate with 15 mL of sterile distilled water, were used to inoculate 500 mL Erlenmeyer flasks containing 50 mL of minimal liquid medium²⁴ with 50% of the nitrogen source (2.4 g/L NH₄NO₃). The flasks were incubated in an orbital shaker at 200 rpm at 28 °C for 4 days. Identical flasks with 50 mL of fresh minimal medium with a 50% nitrogen source were inoculated with 1 mL from the previously mentioned flasks and incubated at 200 rpm at 28 °C for 4 further days. The mycelia were then filtered off and resuspended in a minimal liquid medium²⁴ without NH₄NO₃ (50 mL per 500 mL flask). Immediately afterward, 8 mg of an exogenous substrate, **4**, **5**, or **6** alone, or the **4** plus **5** (8 mg of each) or **4** plus **6**, was added per flask. In all experiments two flasks were incubated without substrate addition as a control for endogenous metabolites (blank). The biotransformations were monitored using thin-layer chromatography, taking daily samples of the culture broth. When the exogenous substrate spot was no longer visible, the incubation was stopped (usually after 4 or 5 days of incubation).

Extraction and Identification Procedures. At the end of each incubation the mycelia were separated from the medium by vacuum filtration, lyophilized, ground with the aid of glass beads, and extracted with EtOAc. The culture broth was also extracted with EtOAc. Aliquots from both extracts were treated with ethereal CH₂N₂ and either bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine or SIGMA-SIL-A (SIGMA) and were analyzed by GC–MS under the following conditions: He as the carrier gas; flow rate of 0.6 mL/min; temperature from 120 to 220 °C increasing at a rate of 5 °C/min, from 220 to 280 °C increasing at 3 °C/min, and kept at 280 °C for 10 min; injector temperature 260 °C; detector temperature 280 °C. In this way, compounds **1**–**12**, GA₁₅ (**15**), GA₂₄ (**16**), and palmitic, stearic, oleic, and linoleic acids were identified initially on the basis of the mass spectra of their derivatives (methyl esters and/or TMS-ethers).⁷ The identification of compounds **4**–**6** was confirmed using our own standards

(see above). The extracts were then chromatographed on a Si gel column eluting with solvent mixtures of increasing polarity. Compounds **1**, **4**–**7**, **9**, **12**, and GA₁₅ (**15**), together with the methyl ester derivative **13a** of the natural acid **13**, were isolated. Thus, the identification of **1**,^{25,26} **7**,^{11,17} **9**,⁸ **12**,^{27,28} and **15**²⁹ could be confirmed by NMR analysis. The chemical structure of the hitherto unknown compound **13** was established in the same way.

Methyl 7 β ,15 α -Dihydroxy-*ent*-kaur-16-en-19-oate (13a): oil; $[\alpha]_D^{25}$ –2.8° (c 0.38, MeOH); IR (film) $\nu_{\max}$ 3426 (OH), 1723 (CO) cm^{–1}; ¹H NMR (CDCl₃, 300 MHz) δ 5.21 (1H, br s, H-17a), 5.09 (1H, br s, H-17b), 4.09 (1H, br s, H-15), 3.94 (1H, t, *J* = 3 Hz, H-7), 3.63 (3H, s, OMe), 2.78 (1H, br s, H-13), 1.24 (3H, s, H-18), 0.81 (3H, s, H-20); ¹³C NMR (CDCl₃, 100 MHz) δ 170.0 (s, C-19), 159.8 (s, C-16), 108.7 (t, C-17), 81.1 (d, H-15), 72.7 (d, C-7), 57.5 (q, OMe), 48.0 (d, C-9), 47.3 (d, C-5), 43.5 (s, C-8), 42.9 (d, C-13), 41.9 (s, C-4), 40.4 (t, C-1), 39.2 (s, C-10), 38.0 (t, C-3), 35.3 (t, C-14), 33.0 (t, C-12), 28.6 (q, C-18), 28.2 (t, C-6), 19.2 (t, C-2), 17.8 (t, C-11), 15.3 (q, C-20); HRFABMS *m/z* 371.2197 (calcd for C₂₁H₃₂O₄Na, 371.2198); EIMS (TMS-ether) *m/z* 420 [M]⁺ (2), 405 (5), 345 (13), 330 (77), 315 (35), 297 (28), 270 (42), 253 (62), 213 (10), 197 (17), 173 (14), 121 (42), 91 (53), 73 (100); (bis-TMS-ether) *m/z* 492 [M]⁺ (9), 402 (48), 387 (7), 343 (4), 312 (4), 297 (5), 253 (14), 221 (7), 181 (10), 156 (13), 147 (25), 91 (16), 73 (100).

Quantitative Analysis. Proportions of compounds **1**, **4**–**6**, **9**, and **13**, as stated in Table 1, were determined on the basis of the peak area of the GC–MS chromatograms, with the aid of calibration curves worked out using solutions of a known concentration. Proportions of the remaining compounds in Table 1 were determined directly from the peak area of the chromatograms.

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Supporting Information Available: Chromatograms of culture broth extracts in feeding experiments with **6** and **6** plus **4**. This information is available free of charge via the Internet at <http://pubs.acs.org>.

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