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THE METABOLISM OF STEVIOL TO 13-HYDROXYLATED *ent*-GIBBERELLANES AND *ent*-KAURANES*

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Key Word Index—*Gibberella fujikuroi* mutant; microbiological conversion; flowering plant gibberellins A_1, A_{18}, A_{19}, A_{20}; 13-hydroxy GA_{12}; bioassays.

Abstract—Steviol (*ent*-13-hydroxykaur-16-en-19-oic acid) is rapidly metabolised by the mutant B1-41a of *Gibberella fujikuroi*. The initial product is the *ent*-7-α-hydroxy derivative which is then further metabolised to gibberellins A_1, A_{18}, A_{19}, A_{20}, 13-hydroxy GA_{12}, the *ent*-6α,7α,13- and *ent*-6β,7α,13(19,6-lactone)-trihydroxykaurenoic acids, and a seco-ring B diacid. This apparently low substrate specificity of the enzymes operative beyond the block in the mutant B1-41a provides a useful model for the biosynthetic pathways to 13-hydroxylated gibberellins of higher plants and a preparative route to these plant gibberellins.

INTRODUCTION

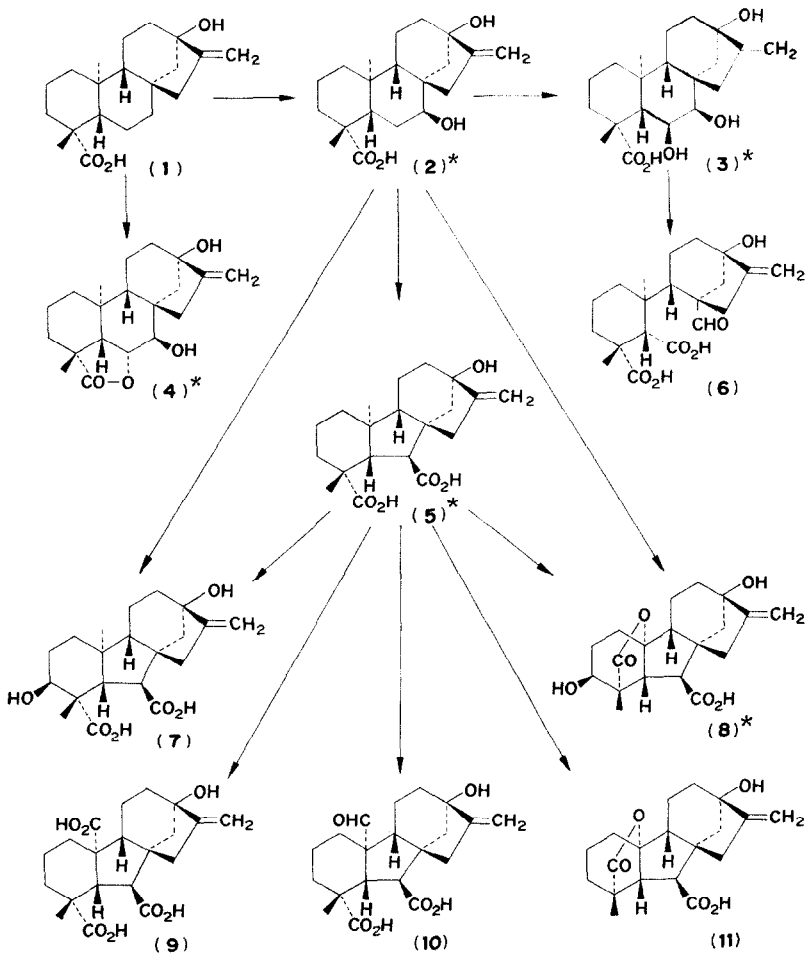
We have previously shown [1] that the mutant B1-41a of the wild-type strain GF-1a of *Gibberella fujikuroi* is blocked for gibberellin (GA) biosynthesis at the step between *ent*-kaur-16-en-19-al (**13**) and *ent*-kaur-16-en-19-oic acid (**14**). We have also shown [2] that the intermediates which are normally present beyond this point in the wild-type parent strain are metabolised by this mutant and we have thus determined metabolic sequences from *ent*-kaurenoic acid (**14**) to the fungal GAs. In a preliminary communication [3] we have extended these metabolic studies with the mutant B1-41a to include non-fungal substrates some of which may be higher plant analogues of the fungal intermediates. In this paper we describe the metabolism of steviol (**1**) (see Scheme 1).

Steviol (**1**) which occurs as the glycoside, stevioside, in leaves of *Stevia rebaudiana* is the 13-hydroxy counterpart of the fungal intermediate, *ent*-kaurenoic acid (**14**) and has been suggested [4] as a precursor of 13-hydroxy GAs in higher plants. Its metabolism by wild-type strains of *G. fujikuroi* has been investigated previously by two groups. Ruddat *et al.* [5] found that [^{14}C]-steviol was metabolised by strain LM-45-399 to an unidentified GA-like compound with properties similar to the then unknown GA_{20}. Hanson and White [6] incubated steviol with strain ACC 917 and isolated 7β,13-dihydroxykaurenolide (**4**).

METABOLISM OF STEVIOL

In initial studies, steviol (**1**) was incubated with resuspended cultures of the mutant B1-41a for 20 hr and for 5 days. The crude acidic extracts of the culture filtrates were analysed by GC-MS with computer data processing as the methyl ester (Me) and methyl ester-trimethylsilyl ether (Me TMS) derivatives. The metabolites, the retention times of the Me esters and the proportion of each metabolite are shown in Table 1. Also identified were GA_{13} (**24**), the seco-ring B diacid (**28**), and GA_3 (**29**) due to the small leak at the metabolic block of B1-41a [1]. However the amount of GA_3

* Part 15 in the series "Fungal Products". For Part 14 see *J. Chem. Soc. Perkin Trans.* 1 721 (1975).



Scheme 1. Metabolism of steviol (1). * Major metabolites.

(**29**) was not greater than that found in controls. Thus neither endogenously produced GA_1 (**8**) nor added GA_1 [2, 7] serve as precursors of GA_3 (**29**). Methanolic extracts of the mycelium from each incubation were analysed by GLC. A trace of steviol but none of its metabolites was present.

Gibberellins A_1 (**8**), A_{18} (**7**), A_{19} (**10**) and A_{20} (**11**) were identified from their published [8] MS. Of the other metabolites those asterisked in Table 1 were identified by isolation and full characterisation and the others by correlation of their MS with those of related known compounds. The evidence for these identifications is presented later.

Before attempting to isolate the metabolites, the effect of incubation time, pH of the resuspension medium and concentration of the substrate were investigated. At pH 4·8 steviol (**1**) was rapidly converted to its *ent*-7-α-hydroxy derivative (**2**) and after 15 min 250 μg of the substrate was completely metabolised by 10 ml of resuspended mycelium to this one product. After 1 hr of incubation, traces of 13-hydroxy GA_{12} (**5**), *ent*-6α,7α,13-trihydroxykaurenoic acid (**3**) and GA_{18} (**7**) were present. After 6 hr GA_1 (**8**) and 7β,13-dihydroxykaurenolide (**4**) were also present. Gibberellin A_{19} (**10**) and the diacid (**6**) were detected after 1 day. All the metabolites except for GA_{18} (**7**) and the kaurenolide (**4**) increased in concentration at the expenses of *ent*-7α,13-dihydroxykaurenoic acid (**2**). Thus in contrast to the metabolism of *ent*-kaurenoic acid (**14**) [2], the *ent*-7α-hydroxy- and *ent*-6α,7α-dihydroxy derivatives (**2**) and (**3**) of steviol accumulated in short term incubations. Jefferies *et al.* [9], also observed the accumulation of *ent*-7α- and -6α,7α-hydroxy derivatives when *ent*-kaurenol hemisuccinate (**17**) was incubated with *G. fujikuroi*.

Steviol (**1**) was completely metabolised between

Table 1. Steviol metabolites*

Metabolite	GLC R_T(min)‡	% of Total 20 hr	% of Total 5 days
13-Hydroxy GA_{12} (5)†	2·1	11·0	18·5
ent-7α,13-Dihydroxykaurenoic acid (2)†	3·0	47·0	0
Gibberellin A_{19} (10)	3·7	0·5	3·5
ent-6α,7α,13-Trihydroxykaurenoic acid (3)† + Gibberellin A_{20} (11)	4·2	9·0	11·0
Gibberellin A_{18} (7)†	4·6	10·0	15·5
Seco-ring B diacid (6)	5·9	0·5	6·0
Gibberellin A_1 (8)†	7·8	11·0	26·0
7β,13-Dihydroxykaurenolide (4)	11·0	6·0	7·5
Other minor peaks		5·0	12·0

* Steviol (2 mg) incubated with resuspended B1-41a mycelium (10 ml) at pH 4·8.
† Metabolites subsequently isolated.
‡ Me ester.

pH 1 and 11 but not at all at pH 13. *ent*-7α,13-Dihydroxykaurenoic acid (**2**) was incompletely metabolised after 5 days except at pH 3 and 5 and at pH 11 it was only slightly metabolised. The most rapid metabolism of this compound (**2**) occurred at pH 5 and so for all further experiments the resuspended medium was buffered at pH 4·8 with KH_2PO_4. All the other metabolites were produced at each pH value between 1 and 11 with some variation in the proportion of each produced at different pH values.

The effect of substrate concentration on the proportion of each metabolite produced after 5 days of incubation of pH 4·8 is shown in Table 2. The yields of all the metabolites except GA_{19} (**10**) did not vary greatly with concentration of substrate. GA_{19} (**10**) increased 16-fold in yield between 10 mg and 0·25 mg of steviol (**1**) incubated with 10 ml of resuspended mycelium.

METABOLIC PATHWAY

With the exception of GA_1 (**8**), the metabolites asterisked in Table 1 were isolated and re-fed to resuspended cultures of B1-41a at pH 4·8. The results are summarized in Scheme 1.

The metabolism of *ent*-7α,13-dihydroxykaurenoic acid (**2**) after 1 and 5 days was practically identical to that of steviol (**1**) except that no 7β,13-dihydroxykaurenolide (**4**) was formed. This contrasts with the production of 7β-hydroxykaurenolide (**19**) from *ent*-7α-hydroxykaurenoic acid (**15**) observed in both cultures [10] and cell-free enzyme preparations [11] of the wild-type strain ACC 917 of *G. fujikuroi*. Oxidation at C-6 may therefore precede that at C-7 in the biosynthesis of 7β,13-dihydroxykaurenolide (**4**) possibly *via* 13-hydroxykaurenolide (**20**). The equivalent step in the normal fungal pathway from kaurenolide (**18**) to 7β-hydroxykaurenolide (**19**) has been ruled out by Hanson *et al.* [10] since they were unable to detect the presence of kaurenolide (**18**) by isotope dilution in cultures to which *ent*-[17-^{14}C]-kaurene had been added.

13-Hydroxy GA_{12} (**5**) was metabolised inefficiently to one main product, identified as GA_{19} (**10**) by GC–MS; traces of GA_1 (**8**) GA_{17} (**9**), GA_{18}, (**7**), GA_{20} (**11**) and an unidentified metabolite were also formed. The unidentified compound formed a MeTMS derivative which had a similar MS to that of GA_3 (**29**) but with different relative intensities of the ions at *m/e* 370 and 208.

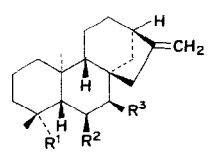
GA_{18} (**7**) was not metabolized when re-fed to B1-41a cultures. This result is surprising in view of the conversion of GA_{14} (**27**) to GA_3 (**29**) by this mutant. With this exception the metabolism of steviol (**1**) to GAs parallels that of *ent*-kaurenoic acid (**14**) [2]. Thus the pathway from steviol (**1**) to 3-desoxy GAs proceeds *via* 13-hydroxy GA_{12} (**5**) while the 3-hydroxy GAs, GA_{18} (**7**) and GA_1 (**8**), appear to be produced by a divergent pathway presumably via the undetected 13-hydroxyGA_{12}-aldehyde (**25**).

When re-fed *ent*-6α,7α,13-trihydroxykaurenoic acid (**3**) was converted in 26% yield to the diacid

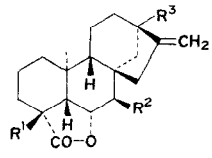
Table 2. Concentration (10 μg/mg substrate)* of metabolites from steviol

Wt fed per 10 ml resuspended mycelium	Steviol (1)	13-OH GA_{12} (5)	*ent*-7α,13-diOH K.A. (2)	GA_{19} (10)	*ent*-6α,7α,13 triOH K.A. (3)	GA_{18} (7)	diacid (6)	GA_1 (8)	7β,13-diOH kaurenolide (4)
10 mg	1	8	41	1	9	7	2	15	16
2 mg	0	17	0	5	14	18	4	33	10
0·25 mg	0	12	0	16	8	18	trace	12	32

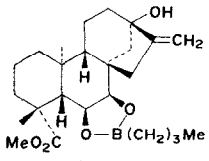
* Determined by GLC.



	R^1	R^2	R^3
(12)	Me	H	H
(13)	CHO	H	H
(14)	COOH	H	H
(15)	COOH	H	OH
(16)	COOH	OH	OH
(17)	CH_2O	H	H
	$CO{\cdot}CH_2CH_2{\cdot}COOH$		



	R^1	R^2	R^3
(18)	Me	H	H
(19)	Me	OH	H
(20)	Me	H	OH
(21)	CH_2OH	OH	H



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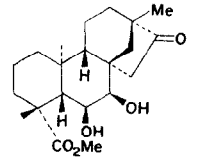

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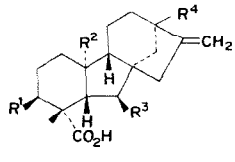
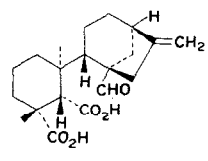


	R^1	R^2	R^3	R^4
(24)	OH	COOH	COOH	H
(25)	H	Me	CHO	OH
(26)	H	Me	COOH	H
(27)	OH	Me	COOH	H



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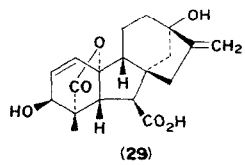


(29)

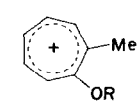
(**6**), identified from the MS of the Me and MeTMS derivatives. This transformation is analogous to that [10, 12] of *ent*-6α,7α-dihydroxykaurenoic acid (**16**) to fujenal, the anhydride of (**28**). A minor metabolite of (**3**) was identifed as an *ent*-x,6,7,13-tetrahydroxykaurenoic acid by GC–MS.

CHARACTERIZATION OF METABOLITES

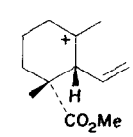
Steviol (**1**) provided a useful model for interpreting the MS of the *ent*-kaurenoid metabolites. The MS of steviol (**1**) and the Me ester contain an intense ion at *m/e* 121 which, in the case of steviol, was shown to be a doublet (3:1, C_8H_9O:C_9H_{13}). The ion $C_8H_9O^+$ and an intense ion at *m/e* 193 in the MS of the MeTMS derivative probably arise from rings C/D and may have the respective structures (**30**) and (**31**) or their benzyl equivalents. [cf Ref. 13] The MS of the MeTMS derivatives of all 13-hydroxy GAs contain [8] the *m/e* 193 ion. The ion $C_9H_{13}^+$ of the *m/e* 121 doublet may have structure (**33**), which is formed [14, 15] from *ent*-kaurene (**12**) and related compounds.

13-HydroxyGA$_{12}$ (**5**) was separated from the other metabolites from a pH 4·8 incubation of steviol (**1**) by means of partition CC on Sephadex LH-20 [16] followed by PLC. The NMR spectrum was similar to that of GA$_{12}$ (**26**) except for the deshielding of one of the C-17 proton signals by 0·5 ppm by the C-13 hydroxyl group. The formation of a *bis*-(monodeuteriomethyl) ester and comparison of the MS of this ester, the *bis*-Me ester, the MeTMS derivative, and the free acid, established the presence of two carboxyl groups and a 13-hydroxy group. These MS were unexceptional, that of the MeTMS derivative contained the ions at *m/e* 193, 207 and 208 characteristic of TMS derivatives of 13-hydroxy GAs.

ent-7α,13-Dihydroxykaurenoic acid (**2**) was isolated from a pH 9 incubation of steviol (**1**). The NMR spectrum was similar to that of *ent*-7α-hydroxykaurenoic acid (**15**) except for the deshielding effect of the 13-hydroxy group on one of the C-17 protons. The MS of the acid, the Me ester, the monodeuterio-Me ester, and the MeTMS derivative were consistent with the structure (**2**); that of the Me ester was very similar to the MS of methyl *ent*-7α-hydroxykaurenoate with M^+, M^+-18, M^+-18-59, and M^+-18-60 shifted to higher mass by 16 a.m.u. The major peaks in the MS of the MeTMS derivative were mass-matched. The ion at *m/e* 193 which also occurs in the MS of Me steviol TMS has the composition $C_{11}H_{17}OSi$ corresponding to the structure (**31**), as suggested above. The ion at *m/e* 167 with the composition $C_9H_{15}OSi$ may be derived from it



(**30**) R = H
(**31**) R = TMS



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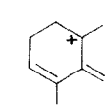

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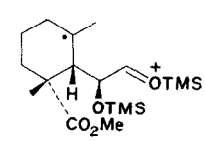
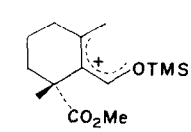


(**34**)



(**35**)

by loss of CH≡CH as has been found [17] to occur with the tropylium (benzyl) ion itself. The published MS of the MeTMS derivatives of all 13-hydroxy GAs contain [8] a peak at *m/e* 167 as well as the *m/e* 193 ion. The base peak at *m/e* 281 in the MS of the MeTMS derivative of *ent*-7α,13-dihydroxykaurenoic acid (**2**) has the composition, $C_{14}H_{25}O_2Si_2$, corresponding to the TMSO-substituted version of the base peak (**31**) in the MS of steviol MeTMS. Thus these peaks at *m/e* 193 and 281 may contain C-7 unless there is a transfer of the OTMS group from C-7. The ion at *m/e* 208 appears to be derived from the cleavage of the 7,8- and 9,10-bonds with the charge residing on rings C/D. The same cleavages with hydrogen transfer and the charge residing on rings A/B would account for the ion at *m/e* 285 which, by elimination, would yield the more intense ion at *m/e* 195 ($C_{12}H_{19}O_2$) with structure (**32**). The loss of HCO_2Me from the ion (**32**) provides the hydrocarbon peak at *m/e* 135 ($C_{10}H_{15}$).

Gibberellins A_{19} (**10**) and A_{20} (**11**) were not isolated. They were identified in the crude extract from a pH 4·8 incubation of steviol (**1**) by GC–MS comparison of the Me and MeTMS derivatives of GA_{19} and of the MeTMS derivative of GA_{20} with the published MS [8].

ent-6α,7α,13-Trihydroxykaurenoic acid (**3**) was isolated from a steviol (**1**) incubation by partition CC followed by PLC. In the NMR spectrum the *ent*-6β(*ax*)-proton was coupled to the *ent*-5α(*ax*)-proton with *J* 10 Hz and to the *ent*-7β(*eq*)-proton with *J* 2 Hz. The C-18 protons were deshielded by 0·4 ppm compared to those of *ent*-7α-13-dihydroxykaurenoic acid (**2**) due to the *ent*-6α-hydroxyl and one of the C-17 protons was deshielded by the C-13 hydroxyl. Comparison of the MS of the acid, the Me ester, and the MeTMS derivative confirmed the presence of one carboxyl and three hydroxyl groups. The MS of the Me ester contained strong peaks at *m/e* 137 and 109, derived by cleavage of the 9,10- and 5,6-bonds [14]. The major peaks in the MS of the MeTMS derivative were mass-matched. The molecular ion showed losses of one and two TMSOH molecules. The ion at *m/e* 372 ($C_{18}H_{36}O_4Si_2$) corresponds to the ion (**34**) formed by cleavage of the 7,8- and 9,10-bonds. The peak at *m/e* 269 is a doublet of equal intensities; one ($C_{14}H_{25}O_3Si$) is probably the ion (**35**) formed by cleavage of the 6,7- and 9,10-bonds and the other ($C_{13}H_{25}O_2Si_2$) appears to be derived from rings C/D. Loss of HCO_2Me from the ion (**35**) would account for the ion at *m/e* 209. The ion at *m/e* 191 ($C_{11}H_{15}OSi$) is a highly unsaturated fragment.

The presence of the 6,7-diol in the metabolite (**3**), indicated [8] by the *m/e* 147 ion in the MS of the MeTMS derivative, was confirmed by the formation of an *n*-butylboronate [18, 19] of the Me ester. This derivative and its mono-TMS were characterized by GC–MS. The molecular ions showed the expected $^{10}B:^{11}B$ ratio of 1:4 and fragmentation patterns.

The Me ester of the metabolite (**3**) was shown to undergo partial decomposition during GLC to the rings C/D rearranged ketone (**23**). The latter was collected by preparative GLC and identified as the TMS derivative by GLC and GC–MS.

Gibberellin A_{18} (**7**) was isolated and identified as the Me ester from the published MS [8].

The seco-ring B diacid (**6**) was characterized by GC–MS of the Me and MeTMS derivatives. The MS of the Me ester showed no M^+ but gave ions at M^+-32, M^+-32-18, M^+-60, and M^+-32-60; it was identical below *m/e* 230 to that of the non-hydroxylated diacid (**28**). The MS of the MeTMS derivative was also similar to that of the MeTMS of the diacid (**28**) but had in addition the fragment ions, *m/e* 208 and 207, typical of a 13-OTMS substituent as well as ions at *m/e* 209 and 237. This acid (**6**) was tentatively but erroneously identified [20] as a metabolite of the wild-type parent GF-1a of the mutant B1-41a.

Gibberellin A_1 (**8**) was isolated, free from the small amount of GA_3 present through the leakage in the mutant, by partition column chromatography [16]. It was identified from the published MS [8] of its Me ester and MeTMS derivative.

7β,13-Dihydroxykaurenolide (**4**) was identified by GC–MS without derivatisation and as the TMS derivative. The MS of the underivatised metabolite showed peaks at *m/e* 137 and 109 typical [14] of kaurenolides unsubstituted in ring A. The MS of the TMS derivative also contained these peaks in addition to the ions at *m/e* 208, 207, 193, and 167 characteristic (see Ref. 8 and above) of 13-OTMS substitution. This compound was previously isolated by Serebryakov *et al.* [21] from another wild-type strain of the fungus, and

Table 3. Lettuce hypocotyl bioassay

Compound (μg/ml)	Length of hypocotyl (cm)*							LSD
	10^{-1}	5×10^{-1}	1	5	10	50	100	5%
GA_3 (**29**)	7·00	11·00	12·05	14·45	13·80			0·8
GA_{12} (**26**)	3·72	4·22	4·11	4·83	4·44	6·11		0·54
13-Hydroxy GA_{12} (**5**)	4·17	4·06	3·79	5·22	4·78	6·06		0·54
ent-7α,13-dihydroxy-kaurenoic acid (**2**)	3·70	4·85	4·25	4·50	5·75		5·6	0·64
ent-6α,7α,13-dihydroxy-kaurenoic acid (**3**)	3·65	3·5	3·55	3·60	3·60		4·5	0·52

* Mean of 10 replicates (mean control value 4·1 cm).

by Hanson and White [6] from incubation of steviol with *G. fujikuroi* strain ACC 917.

BIO-ASSAYS

The biological activities of *ent*-7α,13-dihydroxykaurenoic acid (**2**), *ent*-6α,7α,13-trihydroxykaurenoic acid (**3**), and 13-hydroxy GA_{12} (**5**) in the lettuce hypocotyl [22] and dwarf rice [23] bioassays are shown in Tables 3 and 4 respectively.

The acid (**2**) had low but significant activity in both tests and the acid (**3**) was not significantly active in the lettuce hypocotyl bio-assay and had only very low activity in the dwarf rice assay. 13-Hydroxy GA_{12} (**5**) had similar activity to GA_{12} (**26**) in both tests but this was only 0·1% of GA_3 at the highest concentrations tested.

DISCUSSION

Steviol (**1**) is not an endogeneous metabolite of *G. fujikuroi*. Its efficient conversion by the mutant B1-41a into 13-hydroxylated analogues of the normal metabolites of *G. fujikuroi* is both of theoretical and practical interest. The present studies do not show whether the metabolism of these 13-hydroxylated analogues of the normal fungal metabolites is due to the low specificity of the enzymes of the GA-pathway or due to different, perhaps induced, enzymes. However the metabolism of the higher plant metabolite, steviol (**1**), to 13-hydroxy GAs of higher plants by the fungus provides a useful model for GA-biosynthesis in higher plants. Since steviol (**1**) is reasonably available, its metabolism also provides a practical route to inaccessible plant GAs. For example GA_{18} (290 mg) was originally obtained by extracting 162 kg of immature seeds of *Lupinus luteus* [24]; the same quantity of GA_{18} could now be obtained by incubation with B1-41a of the steviol derived from 130 g of the dried leaves of *Stevia rebaudiana*.

EXPERIMENTAL

General experimental conditions were as described previously [25].

GLC. After derivatization, samples were analysed on a silanized glass column (152 × 0·64 cm) packed with 3% QF-1 on Gas Chrom A (80-100 mesh). N_2 at 75 ml/min at 230°. GC-MS was as described previously [1].

For the formation of *n*-butylboronate derivatives, methylated extracts were dissolved in dry $HCONMe_2$ and a soln of *n*-$BuB(OH)_2$ in $HCONMe_2$ was added until no further change was visible in the GLC trace. For monodeuteriomethylation, extracts were twice dissolved in MeOD and evaporated and then dissolved in MeOD. Alcohol-free CH_2N_2 in Et_2O was added and after evaporation, the extract was dissolved in MeOH and evaporated.

Table 4. Dwarf rice bioassay

Compound (μg/ml)	Length of second leaf sheath $(cm)_2$*								LSD
	10^{-3}	10^{-2}	10^{-1}	5×10^{-1}	1	5	10	50	5%
GA_3 (**29**)	18·6	25·6	44·2		50·5				4·3
GA_{12} (**26**)	20·3	25·3	25·6		26·7		24·4		2·8
13-Hydroxy GA_{12} (**5**)	22·0	27·7	27·9		26·5		29·1		3·1
ent-7α,13-hydroxy-kaurenoic acid (**2**)				23·1	25·7	24·9	27·3	27·0	2·6
ent-6α,7α,13-dihydroxy-kaurenoic acid (**3**)				16·6	16·4	19·2	20·0	22·2	2·2

* Mean of ten replicates (mean control value 17·0 cm).

Culture maintenance and growth. As described [1, 2] except that B1-41a was cultured on 40%-ICI medium (the medium defined by Geissman *et al.* [26] but containing only 40% of the NH_4NO_3).

Resuspended cultures. Mycelium of B1-41a was resuspended in 0%-ICI medium. For the small scale steviol feeds and the re-feeds of steviol metabolites, substrates (2 mg) dissolved in Me_2CO were added to hot sterile H_2O (2 ml) in conical flasks (100 ml). Mycelial suspension (10 ml) was added and incubated at 25° on a reciprocal shaker for the time stated. For the time course experiment steviol (0·25 mg) was fed using these same conditions. To study the effect of substrate concn, steviol (0·25, 2·0 and 10·0 mg) was fed under these conditions for 5 days. For the study of variation in metabolism with pH value, 3-day-old cultures of B1-41a on 40%-ICI medium (140 ml) were filtered and the mycelium was resuspended in 0%-ICI medium (70 ml). 0%-ICI medium (5 ml) containing KH_2PO_4 (27·2 g l) was added to conical flasks (14 × 100 ml) and the medium in pairs of flasks was adjusted to pH values 1,3,5,7,9,11 and 13. After autoclaving, steviol (0·25 mg) in Me_2CO was added to the hot medium in each flask. Resuspended mycelium (5 ml) was added to each flask when cool. One flask of each pH value was incubated for 1 day, the other for 5 days. Work-up of the small scale feeds involved centrifugation (20 min at 2500 rpm.), addition of the supernatant to 1M KH_2PO_4 (5 ml) at pH 2·5 and extraction with EtOAc (10 ml). The extracts were evaporated with a stream of N_2 and derivatized.

Isolation of steviol (**1**). Dried leaves of *Stevia rebaudiana* were extracted with H_2O. The filtrate was extracted with EtOAc at pH 2·5 and pH 9·0, then with *n*-BuOH at pH 9·0. Evaporation and crystallization of this extract gave stevioside, mp 195–8° (lit. [27] mp 196–8°). Stevioside was hydrolysed using "Boots Pectolytic Enzyme" at pH 4·0 to give steviol (*ent*-13-hydroxykaur-16-en-19-oic acid) (**1**) mp 199–200·5° (from Me_2CO–H_2O) (lit. [28, 29] mp 215°, 208–10°); (Found: M^+ 318·220. $C_{20}H_{30}O_3$ requires 318·219); τ ($CDCl_3$) 9·05 (*s*, 20-H_3), 8·77 (*s*, 18-H_3), 5·18 (*br*, 17-H) and 5·02 (*br*, 17-H), *m/e* 318 (M^+, 47%), 300 (22), 272 (12), 260 (17), 254 (13) and 121 (100, 1:1 doublet. Found: 121·065 and 121·102. C_8H_9O and C_9H_{13} require 121·065 and 121·102 respectively); Me ester, *m/e* 332 (M^+, 24%), 317 (2), 314 (8), 299 (5), 274 (13), 273 (21), 272 (8), 254 (13), 121 (100), 109 (26) and 107 (25); as the MeTMS derivative, *m/e* 404 (M^+, 13%), 389 (2), 345 (1), 214 (6), 207 (3), 193 (100), 180 (4), 75 (3) and 73 (13).

ent-7α,13-*Dihydroxykaur*-16-*en*-19-*oic acid* (**2**). Steviol (**1**) (200 mg) in Me_2CO was added to conical flasks (4 × 500 ml) containing hot H_2O (5 ml). Mycelium of B1-41a was resuspended in 0%-ICI medium (200 ml) which has been adjusted to pH 9·0 with 2 N KOH. Portions (50 ml) were added to the flasks containing steviol and incubated for 24 hr. The medium was filtered, adjusted to pH 2·5 and extracted with EtOAc. After evaporation, this extract was purified by PLC on SiO_2 with petrol–EtOAc–AcOH (50:50:1) to give *ent*-7α,13-dihydroxykaur-16-en-19-oic acid (**2**) (177 mg), mp 274–276° (from Me_2CO); (Found: M^+, 334·214. $C_{20}H_{30}O_4$ requires M, 334·214), ν_{max} (Nujol mull) 3440, 1700, 1640, 1165, 1050 and 895 cm^{-1}, τ (C_5D_5N) 8·72 (*s*, 18-H_3), 8·60 (*s*, 20-H_3), 6·06 (*br*, 7-H), 4·92 (*br*, 17-H) and 4·50 (*br*, 17-H), *m/e* 334 (M^+, 9%), 316 (39), 301 (6), 298 (6), 288 (7), 270 (13), 255 (9), 137 (39), 123 (26), 109 (31), 91 (33) and 44 (100); Me ester, *m/e* 348 (M^+, 7%), 330 (36), 316 (5), 312 (6), 271 (27), 270 (21), 238 (11), 137 (34), 123 (100) and 109 (48); MeTMS derivative, *m/e* 492·309 (M^+, 100%. $C_{27}H_{48}O_4Si_2$ requires 492·309), 402 (25), 281·138 (64. $C_{14}H_{25}O_2Si_2$ requires 281·139), 208 (15), 195·139 (20, $C_{12}H_{19}O_2$ requires 105·138), 193·105 (19, $C_{11}H_{17}OSi$ requires 193·105), 167·089 (24, $C_9H_{15}OSi$ requires 167·089), 135·117 (11, $C_{10}H_{15}$ requires 135·117), 75 (32) and 73 (61).

The diacetate, needles mp 250–255° (from Me_2CO–H_2O); (Found: M^+ 418·235. $C_{24}H_{34}O_6$ requires 418·235); ν_{max} 1730, 1670, 1370, and 970 cm^{-1}; τ 9·00 (*s*, 18-H_3), 8·84 (*s*, 20-H_3), 7·98 (*s*, COMe), 7·94 (*s*, COMe) and 5·09 (*br*, 7-H and 17-H_2); *m/e* 418 (M^+, 1%), 376 (1), 358 (28), 316 (7), 298 (46) and 43 (100); Me ester 432 (M^+, 0), 390 (1), 372 (76), 330 (18), 312 (100), 253 (32), 252 (34), 183 (40), 121 (70) and 43 (49).

13-*Hydroxy GA*$_{12}$ (**5**), ent-6α,7α,13-*trihydroxykaurenoic acid* (**3**), *GA*$_1$ (**8**) *and GA*$_{18}$ (**7**). Steviol (**1**) (2·6 g) was distributed between conical flasks (65 × 500 ml) and B1-41a mycelium, re-suspended in 0%-ICI medium (100 ml) was added to each flask. After 7 days incubation the culture filtrate was extracted with EtOAc at pH 8·0 (2 × 6·5 l.) and then at pH 2·5 (3 × 6·5 l.). On evaporation these extracts gave gummy "neutral" (1·25 g) and "acidic" (1·39 g) extracts respectively. GC-MS of the "neutral" extract as the MeTMS derivative showed that the major components were steviol (**1**), *ent*-7α,13-dihydroxykaurenoic acid (**2**) and 7β,13-dihydroxykaurenolide (**4**). The "acidic" extract was purified on the wide range partition column systems [16]. Thus the "acidic" extract, dissolved in aq. phase (30 ml) and adsorbed on Sephadex LH-20 (14 g), was packed on a column of Sephadex LH-20 which had been prepared by swelling the gel (450 g) in aq. phase, packing into a glass column (5 × 90 cm) and eluting with organic phase. The column was eluted with "organic" phase (14 l.) followed by aq. phase (2·4 l.). Fractions (100 ml) were collected and monitored by GLC of methylated aliquots.

Combined fractions 44–67 (190 mg) contained two major compounds, resolved by PLC on SiO_2 using EtOAc–$CHCl_3$–AcOH (15:5:1). Elution of a band at R_f 0·6 gave 13-hydroxy GA_{12} (**5**) (18 mg), mp 261–274° (from Me_2CO–petrol); (Found: M^+, 348·194. $C_{20}H_{28}O_5$ requires 348·194), ν_{max} 3600, 1715, 1620 and 1040 cm^{-1}, τ 8·86 (*s*, 18-H_3), 8·38 (*s*, 20-H_3), 5·81 (*d*, *J* 12 Hz, 6-H), 4·92 (*s*, 17-H) and 4·44 (*s*, 17-H), *m/e* 348 (M^+, 1%), 330 (62), 302 (63), 287 (13), 284 (25), 257 (14), 136 (26), 121 (30), 109 (28) and 44 (100); as the Me ester, *m/e* 376 (M^+, 0·5%), 344 (11), 316 (100), 301 (14), 284 (12), 257 (25) and 241 (11); as the MeTMS derivative, *m/e* 448 (M^+, 34%), 419 (8), 416 (9), 389 (22), 251 (30), 241 (16), 235 (25), 209 (43), 208 (98), 207 (100), 193 (23), 181 (72), 167 (9), 75 (8) and 73 (24). Elution of a band at R_f 0·3 gave ent-6α,7α,13-trihydroxykaur-16-en-19-oic acid (**3**) (78 mg), mp 239–242°; (Found: M^+, 350·209. $C_{20}H_{30}O_5$ requires 350·209), ν_{max} (Nujol mull) 3560, 3500, 3440, 1705, 1665, 1160 and 905 cm^{-1}, τ (C_5D_5N) 8·74 (*s*, 18-H_3), 8·18 (*s*, 20-H_3), 7·58 (*d*, *J*=10 Hz, 5-H), 7·21 (*br*, 15-H_2), 6·04 (*d*, *J*=2 Hz, 7-H), 5·08 (*dd*, *J* 10 Hz and 2 Hz, 6-H), 4·92 (*br*, 17-H) and 4·52 (*br*, 17-H), *m/e* 350 (M^+, 6%), 332 (78), 314 (38), 286 (82), 257 (42), 137 (99) and 109 (100); Me ester, *m/e* 364 (M^+, 1%), 346 (48), 328 (8), 314 (28), 303 (13), 286 (32), 271 (19), 257 (24), 137 (59) and 109 (100); MeTMS derivative, *m/e* 580·344 (M^+, 42%) ($C_{30}H_{56}O_5Si_3$ requires 580·343), 565 (35), 490 (13), 410·231 (6) ($C_{21}H_{38}O_4Si_2$ requires 410·231), 372·214 (12) ($C_{18}H_{36}O_4Si_2$ requires 372·215), 269 (100, 1:1 doublet); (Found: 269·157 and 269·141. $C_{14}H_{25}O_3Si$ and $C_{13}H_{25}O_2Si_2$ require 269·160 and 269·139 respectively), 235·151 (18) ($C_{18}H_{19}$ requires 235·149 or $C_{14}H_{23}OSi$ requires 235·152), 209·135 (35) ($C_{12}H_{21}OSi$ requires 209·136), 191·090 (27) ($C_{11}H_{15}OSi$ requires 191·189), 167·089 (36) ($C_9H_{15}OSi$ requires 167·089), 147 (21) 75 (26) and 73 (94); Me-*n*-Bu boronate, *m/e* 430 (12%), 429 (3), 412 (28), 411 (6), 355 (30), 344 (100), 314 (46), 301 (99), 137 (98), 123 (63) and 109 (73); MeTMS-*n*-Bu boronate, *m/e* 502 (82%), 501 (18), 487 (10), 486 (2), 473 (8), 472 (2), 449 (10), 208 (26), 207 (19), 167 (100), 75 (16) and 73 (46).

Combined fractions 68–80 (66 mg) contained GA_{19} (**10**) which was identified by GC–MS but not isolated.

Combined fractions 83–104 (132 mg) contained GA_1 (**8**) and the diacid (**6**). PLC on SiO_2 with EtOAc–$CHCl_3$–AcOH (15:5:1) and elution of a band at R_f 0·4 gave GA_1 (**8**) (102 mg), mp 240–4° (from Me_2CO–petrol) (lit. [30] mp 256–260°) recognized by the MS of its Me ester [8].

Combined fractions 105–124 (136 mg) were a mixture (GC–MS) of GA_1 (**8**), GA_{18} (**7**) and GA_3 (**29**).

Combined Fractions 125–140 (organic phase) with Fractions 1–4 (aq. phase) (total 120 mg) were purified by PLC on SiO_2 using EtOAc–$CHCl_3$–AcOH (15:5:1) and PLC on SiO_2 using EtOAc–petrol–AcOH (70:30:1) gave GA_{18} (**7**) (63 mg), mp 243–4° (from Me_2CO–petrol) (lit. [24] mp 240–2°) recognized by the MS of its Me ester [8].

7β,13-Dihydroxykaurenolide (**4**) *and the diacid* (**6**). These were identified (GC–MS) in crude extracts. Diacid (**6**) MeTMS, *m/e* 464 (M^+, 3%), 435 (5), 420 (4), 237 (19), 227 (23), 209 (33), 208 (14), 207 (6), 195 (91), 193 (10), 167 (100), 109 (17), 107 (60), 75 (16) and 73 (76); 7β,13-Dihydroxykaurenolide (**4**), *m/e* 332 (M^+, 30%), 314 (16), 257 (16), 152 (66), 137 (64) and 109 (100); TMS derivative, *m/e* 476 (M^+, 100%), 267 (30), 208 (44), 207 (10), 193 (12), 167 (8), 137 (6), 109 (35), 75 (13) and 73 (49).

Bio-assays. The lettuce hypocotyl bio-assays using cv *Arctic*, were performed according to the method of Frankland and Wareing [22]. The rice bio-assay method used was the Tanginbozu Dwarf Rice Bio-assay (micro-drop method) of Murakami [23].

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